A study of the phase transitions of sugars found in chocolate

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Certificate

This is to certify that research work embodied in this thesis entitled "A study of the phase transitions of sugars found in chocolate" has been carried out by myself under the supervision and guidance of Dr. Paul Royall and Dr. Carole Elleman.

Rim Jawad

Abstract

The overall aim of the PhD project was to investigate the phase transitions of chocolate sugars. To achieve this, the chemical purity and the re-crystallisation of both amorphous lactose and amorphous sucrose in a number of model systems were monitored.

Both amorphous lactose and sucrose were prepared by spray- and freeze- drying of aqueous solutions of lactose and sucrose respectively. The amorphicity of the dried samples was successfully confirmed by differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA). NMR analysis was performed and peak areas of partially resolved doublets at 6.3 and 6.6 ppm were used to calculate the percentage (%) of α- and β- lactose present. The β/α anomer contents of amorphous lactose measured by 1H-NMR had standard deviations as low as 0.1% w/w (n = 6). Drying a lactose solution 4 h after its preparation led to almost 35% w/w difference in anomer composition within solid amorphous material compared to samples dried after only 30 min e.g. in freeze-dried samples, the β- content was 60 ± 0.1% w/w (4 h) and 25 ± 1.0% w/w (30 min).

Polarimetery was used not only to investigate the kinetics of mutarotation for lactose solutions at different temperatures but also to confirm the purity of amorphous sucrose by measuring the content of invert sugars, if any. A design of experiments (DoE) approach was applied to investigate the impact of minerals, water vapour and sugar composition on the crystallisation of both sugars. Hot stage microscopy was utilised to monitor the phase changes during crystallisation upon heating.

DoE work showed that a shortage of water during crystallisation of sucrose led to a higher crystallisation temperature T_{crys}. This finding supports the empirical observation from pilot scale chocolate crumb making which states that increasing

pressure, which will increase H_2O in the head space, encourages more crystallisation as the crystallisation temperature is predicted to be lower. Minerals also had an impact on the recipe by reducing the rate of crystallisation at a concentration of 3% w/w NaCl. Lactose present in the recipe interacted with sucrose inhibiting the crystallisation of both sugars. DoE has also proven to be a very efficient methodology, by saving time and resources, to investigate the correlations that may exist among different variables.

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Abstracts

Jawad, R.; Elleman, C.; Royall, P.G. "Characterization of the anomeric composition of amorphous lactose by solution based proton NMR" *American Association of Pharmaceutical Scientists Annual Meeting AAPS* (2011).

Jawad, R.; Elleman, C.; Vermeer, L.; Royall, P.G. "A quantitative measurement of the α and β anomers in amorphous and crystalline lactose" *Royal Society of Chemistry Analytical Research Forum* (2010). Poster abstract promoted to podium presentation.

Papers

Jawad, R.; Elleman, C.; Vermeer, L.; Drake, A.F.; Woodhead, B.; Martin G.P.; Royall, P.G. "The measurement of the β/α anomer composition within amorphous lactose prepared by spray- and freeze-drying using a simple ^{1}H-NMR method" *Pharmaceutical Research* 29: 2 (2012) 511-524.

Jawad, R.; Elleman, C.; Royall, P.G. "The crystallisation of sucrose in the presence of lactose, minerals and residual water" in preparation *Trends in Food Science and Technology* (2013).

Jawad, R.; Drake, A.F.; Elleman, C.; Martin G.P.; Royall, P.G. "The stability of lactose in water: A study of epimerisation at different temperatures" in preparation *Molecular Pharmaceutics* (2013).

Dedicated to my mother and my husband, who are

the sources of inspiration in my life.

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Chapter one – General introduction

Although many of the properties of chocolate are well understood, there is limited data on its purity and the properties of chocolate sugars (lactose and sucrose) which potentially influence the overall behaviour e.g. crystallisation within the chocolate matrix. Therefore, investigating both the chemical and physical purity of lactose and sucrose forms the main thrust of the thesis presented here.

This chapter will briefly introduce chocolate, its history and its ingredients. This will be followed by an overview of the sugars used in chocolate, their role, chemical properties, physical state and purity, with a review of the analytical techniques utilised for their characterisation.

1.1 Chocolate history

Chocolate is one of the most popular confectionery products being sold worldwide. A recent UK statistics study has reported that chocolate-based products represent one of the UK consumer's favourite purchases, PRA15840 UK national statistics (www.statistics.gov.uk). Moreover, chocolate is a key ingredient for a wide variety of products such as biscuits, cakes, confectioneries, hot drinks and snacks (Knapp, 1920; Minifie, 1979).

Cocoa plantations were firstly known and established by the Maya in the lowlands of South Yucatan (Beckett, 2000). Cocoa trees were grown by the Aztecs of Mexico and the Incas of Peru. The Maya and Aztecs mixed ground cacoa seeds with various seasonings to make a spicy, frothy drink. Moreover, in Central America forests, the tropical mix of high rain fall combined with high year round temperatures and humidity resembled the ideal climate for cultivation of the cacoa tree (Beckett, 2000; Minifie, 1979). Thereafter, chocolate has undergone an important development from spreading first as a chocolate drink to the chocolate bar known nowadays. In order to illustrate the development of chocolate from antiquity to the present day, table 1.1 has been included to show and map the key milestones of chocolate and its production

Table 1.1 Important milestones in chocolate history (Beckett, 2000; Knapp, 1920; Minifie, 1979).

1520	Chocolate drink was first introduced to Europe by the Cortez (to Spain in 1520; Italy in 1606; France and United Kingdom in 1657).
1727	Milk was added to the chocolate drink by Nicholas Sanders.
1828	The Dutch developed the cocoa press to remove part of the cocoa butter by Van Houten.
1824	John Cadbury started selling cocoa and drinking chocolate. He prepared them by breaking up the cocoa beans with a pestle and mortar and adding common contemporary ingredients such as treacle and starches.
1831	John Cadbury's cocoa and drinking chocolate was getting more and more popular. John Cadbury established a small factory to become a 'manufacturer of drinking chocolate and cocoa'. It was the start of Cadbury as known today.
1847	Establishment of the first British factory in Bristol/UK to produce the first bar of chocolate by Joseph Fry.
1876	The first milk chocolate was made in Switzerland by Peter Daniel. A well-dispersed chocolate with less fat was produced. During the manufacturing process, chocolate was dried to reduce moisture which causes a poor shelf-life.
1880	Conche machines were introduced in Switzerland by Rodolphe Lint to produce smoother and better tasting chocolate. A conche machine has a granite trough and a roller to break the agglomerates and large particles.
1905	Cadbury launched the world-famous dairy milk bar, and it's still going strong over 100 years later.
1913	Swiss confectioner Jules Sechaud invented a machine for making filled chocolates in Montreux.
1930	The first white chocolate was made. Many of the chocolate products and companies were developed and have remained popular to this day.
After 1930 and throughout the 20th century, the number of chocolate products rapidly increased. However, the scientific publications on whole chocolate diminished due to the high competition among the different chocolate manufacturers as chocolate had become a huge business, and details of new bars were kept top secret to stop rivals copying them!	

1.2 Chocolate ingredients

Chocolate can be described as a dispersion of very fine solid particles in a fat phase (Beckett, 2000). The essential raw materials used for chocolate manufacture are: cocoa mass, sugar, cocoa butter, milk (in milk chocolate), emulsifiers (lecithin) and flavourings (Beckett, 2000). In a powder based chocolate, the basic ingredients are added to each other and are mixed for hours until a smooth thick sugar/cocoa mixture known as "chocolate paste" is produced. An emulsifier is then added to reduce the viscosity and allow the product to be moulded.

Some milk chocolates use crumb as the starting process during chocolate manufacture. Chocolate crumb is a powder consisting of cocoa mass, agglomerates of sugar crystals held together by amorphous sugars and proteins containing isolated droplets of fat (Beckett, 2000). The mixing of the crumb ingredients is usually performed at high temperatures (up to 124 ºC) to achieve a moisture content of chocolate crumb that varies between 0.8% w/w and 1.5% w/w. The brown colour and the caramel type flavour of the crumb is produced by the Maillard reaction that takes place due to the availability of heat, moisture, lactose and proteins during the chocolate crumb manufacture (Beckett, 2000).

The chocolate making process utilizes high grade refined sugars. Traditionally, chocolate has been manufactured containing about 50% w/w sugars; mostly in the form of sucrose. In addition, milk chocolate always contains lactose from the milk components. However, fructose for diabetics' chocolate or non-sugar bulk sweeteners for low calorie chocolates are rarely included (Beckett, 2000).

1.3 Milk

Milk is one of the main ingredients involved in the production process of milk chocolate and chocolate crumb. The whole milk powder content can reach up to 23% w/w of a milk chocolate bar (Table 1.2).

Table 1.2 Typical percentages of components in milk chocolate formulations (Fryer *et al.*, 2000).

Component	Milk chocolate (%w/w)
Cocoa mass	11.8
Added cocoa butter	20.0
Sucrose	48.7
Lecithin	0.4
Flavour compounds	0.1
Whole milk powder	19.1
Total fats	31.5

Cow's milk is used in the production of milk chocolate. It contains lactose, fat, protein and minerals (ash) (Table 1.3).

Table 1.3 Average composition of cow's milk (Varnam *et al.*, 1994).

Component	Percentage	Percentage of solids
Lactose	4.8	37.5
Fat	3.7	28.9
Protein (Casein, whey protein)	3.4	26.6
Non-protein nitrogen	0.19	1.5
Ash	0.7	5.5

The concentration of lactose in milk varies between 4.2% w/v and 5% w/v (Varnam *et al.*, 1994). It is produced by crystallisation from whey powders (Figure 1.1). Lactose will be further discussed in detail in section 1.4.1.

Casein is the principal component of cheese while whey proteins are dairy proteins that are considered as by-products of cheese making. Whey is the watery component removed after the setting of the curd in cheese manufacture (Varnam *et al.*, 1994). The whey waste stream undergoes processing to obtain its final products as lactose, whey powder or demineralised whey powder (Figure 1.1).

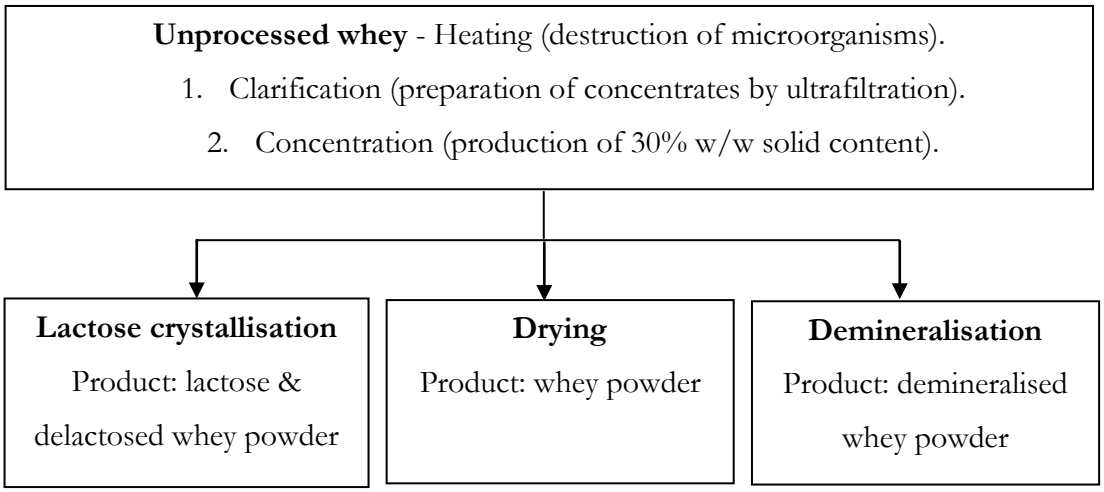

Figure 1.1 Production of whey protein products (modified from Varnam *et al.*, 1994). The production process involves either lactose crystallisation, drying or demineralisation of whey

Up until recently, whey powder was considered a cheap commercial product. However, it has become fairly expensive due to the cost increases of its production. This is a market driven cost increase as demand has risen for proteins which are useful for body building as well as baby milk. Thus, whey permeate was introduced into the production of chocolate. Whey permeate is obtained by the removal of the protein from the whey by precipitation, filtration or dialysis.

Casein is made up of several proteins including α-casein, β-casein and k-casein. It also contains inorganic minerals and citric acid, which all combine together to form an aggregate called 'casein micelle' (Park, 2009). Casein micelle is generally spherical in shape, and contains smaller micelles having similar proportions of k-casein (Park, 2009).

Milk also contains minerals (milk salts) which include various cations and anions like calcium, potassium, sodium, magnesium, phosphate, citrate, chloride and sulphate (Fox *et al.*, 1998; Varnam *et al.*, 1994) (Table 1.4). Minerals can strongly interact with casein, most notably calcium phosphate (Varnam *et al.*, 1994).

Minerals are considered as normal constituents of whey powder (Nijdam *et al.*, 2007). The more acidic the whey, the higher the mineral content is. This may cause off-flavours in the chocolate product and accounts for certain processes favouring the use of demineralised whey

powders (Beckett, 2000). The mineral salts content of milk has been reported in literature (Rodriguez *et al.*, 2001; Fox *et al.*, 1998) (Table 1.4).

Table 1.4 Distribution of milk salts (Fox *et al.*, 1998).

Species present within salts	Content (mg/L)	Form
Sodium	500	Completely ionised
Potassium	1450	Completely ionised
Chloride	1200	Completely ionised
Sulphate	100	Completely ionised
Phosphate	750	10% w/v bound to Ca and Mg 51% w/v $H2PO^-$ 39% w/v HP
Citrate	1750	85% w/v bound to Ca and Mg 14% w/v $citrate^{3-}$ 1% w/v $Hcitrate^{2-}$
Calcium	1200	35 % w/v Ca^+ 55 % w/v bound to citrate 10 % w/v bound to phosphate
Magnesium	130	Similar to calcium

Milk contributes two major components, which are lactose and minerals that could influence sucrose crystallisation and therefore the crumb manufacturing process. This concept will form the basis of chapter 5 which will focus on the impact of these two parameters on the sugar performance and its physical behaviour during the crumb manufacture.

1.4 Chocolate sugars

The project will focus on sucrose and lactose, as they form a major component of the crumb. The crystallisation of fats, their contribution to the chocolate manufacturing process and the chocolate quality produced have been covered in depth in literature (Ziegleder *et al.*, 1990; Loisel *et al.*, 1998; Tietz *et al.*, 2000; Sato, 2001; Attaie *et al.*, 2003; Foubert *et al.*, 2004; Vereecken

et al., 2007). Whereas, the crystallisation of sugars (ingredients that constitute up to 50% w/w of the chocolate formulation) and its impact on the chocolate manufacturing process are still not well explored. Early studies on sugars did not produce a clear understanding of the physical changes that occur during the chocolate manufacturing process (Gloria *et al.*, 2001). Most manufacturers are still using their chocolate recipes without fully understanding the impact that sugars may have on the chocolate formulation. Hence, the importance of this work is to develop an area which has not been fully explored yet and find the appropriate techniques and methods to produce, detect and when required quantify the chemical and physical purity of the sugars utilized in the chocolate manufacturing process.

During powder chocolate manufacture, sugar (sucrose), milk solids (including lactose) and cocoa liquor are mixed (with an overall fat content of 8 - 24% w/w) to form a paste; the mixture then undergoes refining by a five-roll refiner to reduce the particle size from 100 - 950 μm for sucrose and 50 - 400 μm for lactose, to < 30 μm and ˃ 18 μm. This is because a particle size of greater than 30 μm will produce chocolate with a gritty feel in the mouth while a particle size < 18 μm will lead to a highly viscous product. This increase in viscosity is owing to the increase in the total particle surface area. Therefore, the final particle size is a critical parameter since it decides the rheological and sensory properties of the chocolate product (Beckett, 2000).

Milk was originally added to the chocolate drink in 1727. The incorporation of milk into chocolate as 'milk chocolate' started in the 1876 and at that time, only liquid milk was available. To accommodate this, and the fact that milk was a very seasonal product, a process was developed whereby the milk and the chocolate solids were cooked and dried down together as a 'crumb' with a low water activity and an extended shelf life. This had the advantage of also allowing the effective storage of the milk for use during the season where the supply of milk tailed off. Crumb chocolates have distinctive sensory properties compared with powder chocolates and the process is still used today.

During crumb manufacture, starting from liquid milk, the sugars contained in the mass go through several transitions. The lactose is initially in solution and the added granulated sugar (sucrose) will in some processes also be at least partially dissolved. As the water is removed from the mixture, the two sugars become increasingly concentrated. The interaction of the two sugars with each other and the minerals in milk fraction, as the final, principally crystalline product is formed, is the basis for this study.

Sugars are considered the simplest members of carbohydrates. Their chemical structure may be described as an aldehyde or a ketone with multiple hydroxyl groups (Mathlouthi *et al.*, 1995). Sugars are classified as reducing and non-reducing sugars. Reducing sugars have a free carbonyl group either an aldehyde or a ketone. Therefore, all monosaccharides that are not substituted at the anomeric centre (C1 in aldoses and C2 in ketoses) are reducing sugars. However, oligosaccharides can be either reducing or non-reducing. An example of a reduction process is the Maillard reaction which is a chemical reaction between the reactive carbonyl group of the reducing sugar and the nucleophilic amino group of the amino acid in an alkaline medium. It is responsible for changes in colour and flavour in food (Berg *et al.*, 2007).

Sugars are linked to one another by O-glycosidic linkage to form disaccharides and oligosaccharides. Sucrose and lactose are considered as the most abundant disaccharides formed. Sucrose consists of α-glucose and β-fructose joined by a glycosidic linkage between their anomeric carbon atoms. Lactose consists of galactose joined to glucose by a β-1,4 linkage (Berg *et al.*, 2007).

1.4.1 Lactose

Lactose is a disaccharide that consists of β-D-galactose and α/β-D-glucose fragments bonded through a β-1,4 glycosidic linkage (Figure 1.2). Lactose is a white solid carbohydrate with the general formula $C_{12}H_{22}O_{11}$ and a molecular weight of 342.29 g/mole.

Lactose exists in one of three crystalline forms which are α-lactose monohydrate, α-lactose anhydrous and β-lactose anhydrous or in an amorphous state which could alter between α- and β-forms (Figure 1.2). Lactose also can exist in different molecular compounds of α- and β- in different stoichiometric ratios (Figure 1.3). The α- and β- anomers of lactose exhibit two different molecular structures due to the difference in the orientation of hydrogen and the hydroxyl group on carbon atom no. 1.

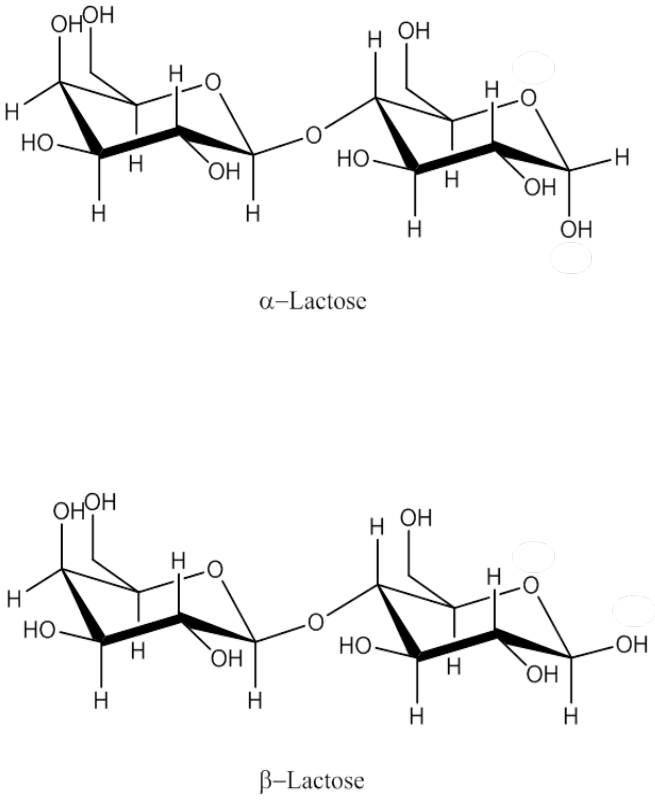

Figure 1.2 Lactose molecule drawn using Chemdraw®.

Both α- and β-lactose can undergo an epimerisation phenomenon (commonly referred to as mutarotation) in which they change into one another until they reach a final equilibrium, in aqueous solution at room temperature, at a ratio of 37 % α-lactose and 63% β-lactose (Fox *et al.*, 1998).

The α- and β- anomers of lactose have distinct solubility values. The solubility of α-lactose monohydrate is 7 g/100 mL whereas the solubility of β-lactose is 50 g/100 mL. Therefore, the β- anomer dissolves faster than the α- anomer which explains the difference in sweetness between both forms (Beckett, 2000; Fox *et al.*, 1997).

As mentioned above, the initial solubility of α- lactose monohydrate is 7 g/100 mL. However, once α-lactose monohydrate is put into a solution, an increasing solubility with time is observed due to the epimerisation. The solution is initially supersaturated with α-lactose yet as the α- anomer is converted to the β- form, the solution becomes unsaturated with respect to α-,

thus more α- dissolves. This process continues until the final equilibrium is achieved and the final solubility of lactose is established as 17 g/100 mL (Fox *et al.*, 1997).

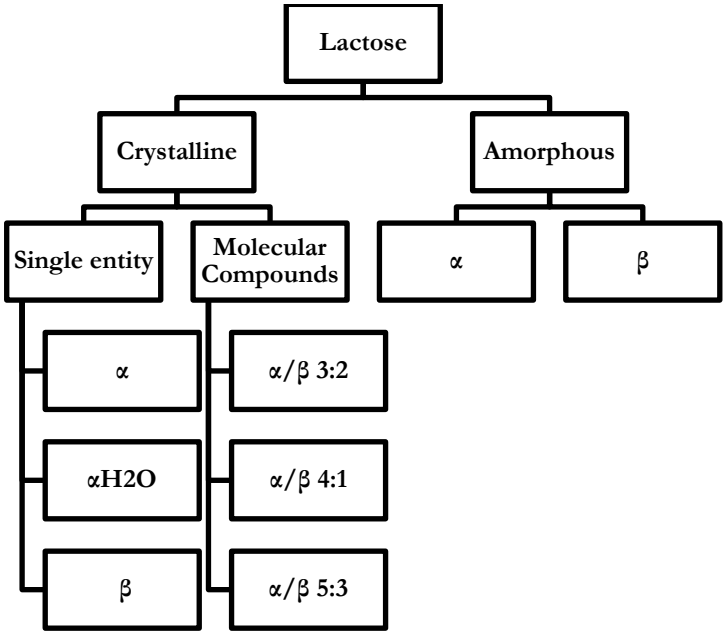

Figure 1.3 Solid-state forms of lactose (proposed by Jarring, personal communication Astrazeneca; Drapier-Beche *et al.*, 1999).

Drapier-Beche *et al.* (1999) was able to differentiate among the different forms of lactose by measuring their melting points as well as their heat of melting by DSC (differential scanning calorimetry) (Table 1.5).

Table 1.5 Melting points (°C) and heat of melting (J/g) of different lactose solid forms (Drapier-Beche *et al.*, 1999).

Lactose Crystalline Form	Melting Point (°C)	Heat of melting (J/g)
α-Lactose Monohydrate	215 ± 2	134 ± 4
β-Lactose	225 ± 3	198 ± 3
Anhydrous α-Lactose	216 ± 2	122 ± 5
5α-/3β- Lactose	209 ± 1	125 ± 4
3α-/2β- Lactose	291 ± 2	159 ± 3

Therefore, the chemical purity of lactose (in terms of its different α- and β- anomers) should be investigated as these differences in their physical properties; solubility and sweetness are considered a significant source of variations and can cause batch-to-batch irreproducibility.

1.4.2 Sucrose

Sucrose is a white solid carbohydrate with the general formula $C_{12}H_{22}O_{11}$ and the molecular weight of 342.30 g/mole (Figure 1.4).

Sucrose is a non reducing dextrorotatory disaccharide (i.e. it rotates plane polarized light clockwise) with a melting point of 186 °C, density = 1.587 g/cm³ and solubility in water of 200 g/100mL at 25°C (Mathlouthi *et al.*, 1995).

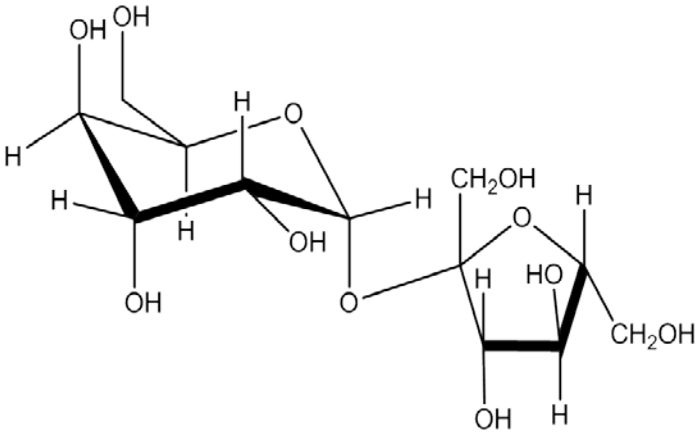

Figure 1.4 Sucrose molecule drawn using Chemdraw®.

In solution, sucrose can be hydrolysed to produce an invert sugar mixture (glucose + fructose). This hydrolysis may be aided by the enzyme invertase or by dilute acid (Garg *et al.*, 2008). In aqueous solution, sucrose has the property of rotating the plane of linear polarized light; the angle of rotation is proportional to the concentration and to the path length of the solution traversed by the light beam (Gergely, 1989).

Unlike lactose, sucrose does not have a free anomeric carbon (as it is not a reducing sugar). Thus, sucrose does not mutarotate and does not have different anomers. Therefore, sucrose is always presented in only one crystal form and consequently one single amorphous form.

1.5 Crystallisation

Crystallisation is a process of formation of solid crystals from a solution, a melt or from amorphous form. Crystallisation process consists of two main steps which are nucleation and crystal growth (Markov, 2003; Kashchiev, 2000; Buckmaster, 1858). Nucleation is often the decisive step in the crystallisation process. It occurs when the solute molecules in a solution start to form clusters which in turn form nuclei (Hartel *et al.*, 1991). Once the nucleation step has been overcome, nuclei grow into macroscopic crystals. Being the driving force for the crystallisation, supersaturation will enhance the competition between nucleation and growth for the solute. Hence, neither nucleation nor growth will predominate over the other, producing crystals with different sizes and shapes (Schmitt *et al.*, 1998; Rodriguez-Hornedo *et al.*, 1999).

Crystalline molecules have regular and well-defined molecular packing. Different polymorphic forms exhibit different thermodynamic properties such as melting point, vapour pressure and solubility. Moreover, crystalline hydrates can be formed in which the water molecule fits in the structural voids and links the molecules into stable crystal structure by H-bonding (Vippagunta *et al.*, 2001).

The rate of crystal growth can be influenced by any impurities or additives present in the solution. It has been reported that common monosaccharides can significantly reduce the overall crystal growth kinetics of sucrose, even leading to a smaller mean particle size of the crystals produced (Ouiazzane *et al.*, 2008). Furthermore, hydrophilic impurities do strongly influence the rate of growth of sucrose crystals as well as their shape, as reported by Belhamri *et al.* (2004). Kubota *et al.* (2000) referred the impact of impurities on sucrose crystallisation to changes of the rate-determining process. Moreover, the rate of crystal growth of lactose can be affected too, by the presence of impurities, sucrose or β-lactose (Hunziker *et al.*, 1927).

In melted chocolate, the primary crystallisation of the cocoa butter takes place at the wall of the container in the form I modification during the tempering stage. These primary unstable

crystals are transported into the bulk by scraping the wall, and during transport into the bulk they will re-crystallise into the stable form V modification (Figure 1.5) (Kattenberg *et al.*, 1989).

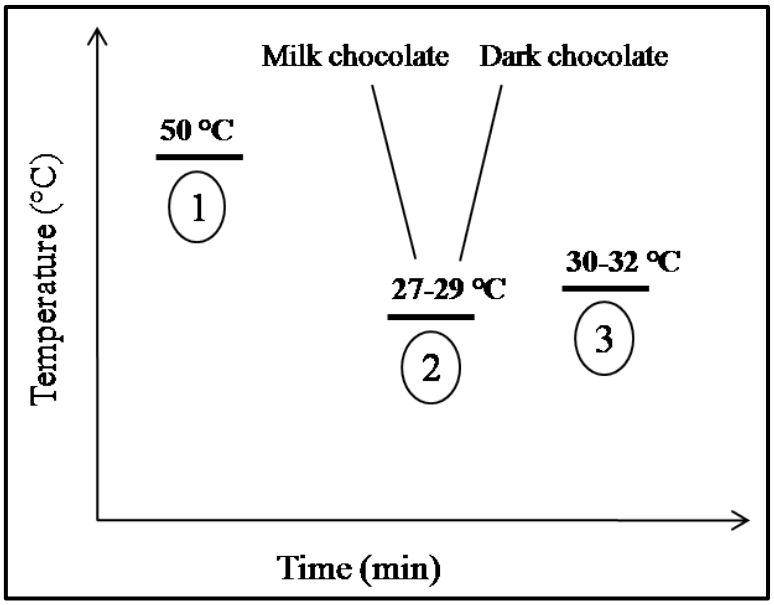

Figure 1.5 The temperature profile of chocolate tempering. Stage 1: chocolate has to be heated up to 45-50 °C, to melt out all existing fat crystals. Stage 2: a decrease of temperature to 27-29 °C plus high shearing: supercooling formation of both stable and unstable crystal nuclei. Stage 3: an increase of temperature to 30-32 °C to melt out most of the unstable forms.

Sugars (including sucrose and lactose) can also crystallise and build their own crystal structure in the chocolate mass which does not solubilise at 30 to 35 ºC. As a consequence, the liquid cocoa butter with the flavour will not be released in the mouth during eating, reducing the sensation of the chocolate flavours (Kattenberg *et al.*, 1989). Therefore, it is important to detect the stage of the manufacturing process at which the crystallisation of the sugars is taking place. The detection and characterisation of the crystallised sugars may subsequently act as a guide to their effect on the quality of the final chocolate product.

1.6 Amorphous state

Due to milling and grinding, there is potential for amorphous sugars to form (Lefort *et al.*, 2004; Aulton, 2002). Amorphous materials are generally brittle and transparent solids which are commonly referred to as *glassy* solids. Typically, amorphous solids are disordered with any long range order; they only exhibit a short-range order over few molecular dimensions. They have different physicochemical properties from their corresponding crystals such as chemical reactivity, molecular mobility, lattice disorder, solubility and water sorption capacity (Yu, 2001; Willart *et al.*, 2008). Amorphous materials are hygroscopic, viscous, cohesive and difficult to flow and disperse (Chiou *et al.*, 2008). Amorphous powders possess greater pore size and volume; hence, they exhibit greater sorption capacity when compared to their crystalline counterparts (Trivedi *et al.*, 2001).

Cooling a material quickly from its molten or liquid form is a common method for generating the amorphous material. As cooling reduces the molecular mobility of the liquid, an amorphous solid is formed when the cooling rate is faster than the rate at which molecules can organise into their crystalline state. Therefore, in order to obtain glassy materials, the solids are heated above their melting point and rapidly cooled through it (Craig *et al.*, 1999). Amorphous materials can also be produced by milling, spray-drying and freeze-drying (Jawad *et al.*, 2012). Techniques like milling and grinding, create disorder, either by divergently breaking the particles up and creating disorder, or by convergently bringing or forming the particles rapidly together, so fast that crystallisation is prevented (Jawad *et al.*, 2012; Langrish, 2009; Haque *et al.*, 2005; Lefort *et al.*, 2004; Surana *et al.*, 2004; Craig *et al.*, 1999; Roos, 1997). The production of amorphous materials (including sucrose and lactose) will be discussed in detail later in this chapter.

Chichester *et al.*, 1988, Markower *et al.*, 1956 and Niediek *et al.*, 1980 had previously given a detailed account on the amorphocity of the sugars which constitute the chocolate crumb. It was claimed that upon milling sucrose, the amorphous form was produced and moisture was

absorbed from the surrounding environment leading to sucrose re-crystallisation. Amorphous sugars have a significant impact on the flavour, flowability and thermal properties of chocolate. A more intense flavoured chocolate can be produced by using amorphous sugars instead of their crystalline counterparts as amorphous sugars possess a very reactive surface and can easily absorb any flavours in the proximity (Beckett, 2000). Moreover, amorphous sugars influence the mouth feel as they readily absorb water giving chocolate a much more cohesive mouth feel. During chocolate processing, amorphous sugars can re-crystallise by absorbing water from the surrounding environment, other chocolate ingredients and humid atmosphere.

In powder based milk chocolate, amorphous lactose (produced by spray-drying of milk) forms a matrix in the milk powders in which proteins, fat globules and air cells are dispersed (Gaonkar *et al.*, 2006). For this reason, manufacturers tend to use skimmed powders and added milk fat so that none of the fat material is trapped in the milk powder matrix and is free to contribute to processing and creaminess flavours.

1.6.1 Glass transition (T_g)

Glass Transition, T_g is the most defining characteristic of amorphous systems. When a solid is heated above its melting point and then rapidly cooled, it forms a supercooled liquid. Depending on the cooling rate, the supercooled liquid can undergo a heat capacity change known as glass transition T_g (Kerc *et al.*, 1995). Therefore, a T_g can be defined as the midpoint in temperature between a supercooled liquid and a brittle amorphous glass when an amorphous material is either heated or cooled. Several studies have shown that the glass transition temperature of an amorphous solid may influence both the chemical and physical stability. In addition, T_g represents a change in the viscoelastic properties of the amorphous material. Below T_g, the glass is hard with high viscosity while above T_g, the glass exhibits low viscosity (Liu *et al.*, 2007; Quintas, 2007).

T_g may vary according to the method by which the amorphous materials were formed. Other driving parameters that affect T_g are cooling rate and experimental heating rate (Kioke, 1995), sample geometry and dimensions (Mayes *et al.*, 1994) and sample purity (Her *et al.*, 1995). Moreover, experimental T_g can be affected by the analytical technique used for evaluation. This is due to the fact that different analytical techniques have different sensitivities with respect to different speeds of molecular motions (Hancock *et al.*, 1997).

T_g is considered roughly as 2/3 to 4/5 of the melting point (Yu, 2001). It was proven in literature that compatible blends of amorphous materials exhibit a single T_g that is intermediate to the individual T_g values of the component materials (Hancock *et al.*, 1994). The T_g of blends should be considered carefully in the project as there will be the potential of preparing amorphous mixtures of sucrose and lactose, forming a single amorphous phase; thus, the T_g of this single amorphous phase is expected to lie between the T_g values of both sucrose and lactose (Hancock *et al.*, 1994).

1.6.2 Plasticisation

Plasticisation is caused by absorption of certain additives to lower the T_g of amorphous solids. Water acts as a potent plasticiser for amorphous and partially amorphous molecules by increasing the molecular mobility and lowering the glass transition temperature T_g (Hancock *et al.*, 1994). Hancock *et al.* (1994) attributed the plasticising effect of water to three main parameters which are the T_g of the amorphous solid, the density and the tendency of the solid to interact with water from its surroundings. These parameters are directly linked to the structure, free energy and free volume of amorphous solids. Roe *et al.* (2005) reviewed the T_g values of sucrose which ranged from 52 °C to 75 °C. They reported the inaccuracy in T_g of sucrose versus moisture which may arise from failure to remove all water content from the dry samples. The T_g of sucrose falls by more than 12°C as the moisture content increases from 0% to 1% w/w (Roe *et al.*, 2005).

1.6.3 Stability and re-crystallisation

Amorphous solids are metastable and tend to become sticky at 10 - 20 °C above T_g, causing caking (Chiou *et al.*, 2008). Hence, they may re-crystallise or express structural relaxation. Amorphous materials are mechanically and/or thermally unstable (Yu, 2001). This is due to their nature that exhibits higher energy, entropy and free energy than their corresponding crystalline materials. Pikal *et al.* (1978) deduced that amorphous molecules have higher internal energy and a greater chemical reactivity when compared to their corresponding crystalline molecules.

Re-crystallisation is a phase transition that takes place in amorphous products when the water content or temperature exceeds a critical value (Roos, 1995). Low molecular weight glass formers, such as drugs and sugars often undergo re-crystallisation at moderate moisture content. The existence of small amounts of absorbed water (at low levels of moisture) can lower T_g, increase the molecular mobility and lead to the re-crystallisation of the amorphous material. The internal structure of amorphous powders is disordered and moisture acts as a lubricant to allow crystallisation of the powders to occur (Chiou *et al.*, 2008). Chichester *et al.* (1988) referenced previous work describing the re-crystallisation of sugars in chocolate due to the instability of amorphous sucrose in the presence of water.

Kedward *et al.* (2000) demonstrated the tendency of sucrose and lactose to re-crystallise at low water content (1% w/w for sucrose and 5% w/w for lactose). More related work has been done on milk chocolate samples by Zeigler (1999). Zeigler and co-workers detected the elimination of glass transition corresponding to lactose by DSC in refined milk chocolate after conching (agitation of all chocolate ingredients together) at 95 °C. As dark chocolate does not contain lactose, no glass transition was detected. This was proved by comparing the refined milk and dark chocolate which revealed that before conching, milk chocolate exhibited a T_g which was eliminated upon conching. However, no T_g was detected with dark chocolate in both stages i.e. before and after conching.

The maximum rate of crystallisation usually takes place between the melting point T_m and above the glass transition T_g (Figure 1.6). If a sample is stored below T_g, the risk of devitrification (the process of crystallisation of an amorphous glass) is considerably reduced due to the low chance of crystal growth caused by the low mobility of the molecules (Craig *et al.*, 1999).

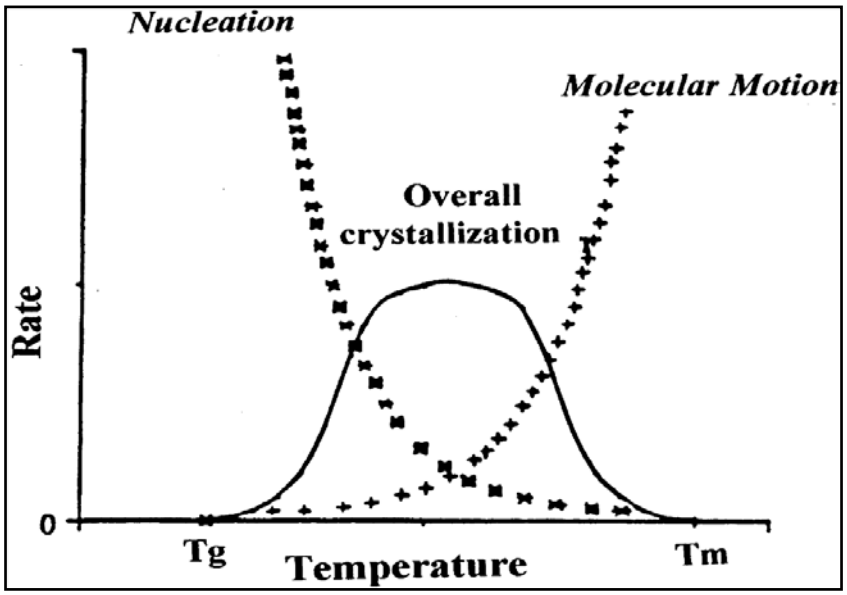

Figure 1.6 A schematic representation of the temperature dependence of the crystallisation process for an amorphous system (Craig *et al.*, l999).

1.7 Production of the amorphous state

Amorphous sugars, as previously discussed, can affect the flow properties and the stability of chocolate. Therefore, it is vital to understand and control how they are produced, stored and analysed. Several techniques have been utilized for the production of amorphous molecules. These techniques are most commonly used in industry to induce the amorphous character into food and pharmaceutical systems (Jawad *et al.*, 2012; Langrish, 2009; Haque *et al.*, 2005; Lefort, *et al.*, 2004; Surana *et al.*, 2004; Craig *et al.*, 1999; Roos, 1997). These methods include:

- Supercooling/quenching of the melt.
- Mechanical Activation of a crystalline mass (e.g. milling, grinding, wet granulation).

- Rapid precipitation from solution (e.g. freeze-drying and spray-drying).

1.7.1 Supercooling/Quenching of the melt

Quenching can be employed for the production of glassy amorphous solids. The process is performed by heating up the material above its melting point to transform it into the liquid state where it loses its crystalline lattice. This stage is followed by rapid cooling below the melting point in which it forms a supercooled liquid. Further cooling causes the formation of the glassy state (Craig *et al.*, 1999). Rapid cooling is too fast for the crystallisation to take place which results in a discontinuity in enthalpy and volume. As a result, a dramatic reduction in the translational and rotational motions of the molecules is observed (Craig *et al.*, 1999). This technique cannot be applied to produce amorphous sucrose as heating up the sample above its melting point will cause melt degradation (Lee *et al.*, 2011) and form a brown product called caramel. This is the case with most saccharides; therefore, supercooling from the melt will not be considered further in the project.

1.7.2 Spray-drying

The process of spray-drying has been widely used in both food and pharmaceutical manufacturing processes. Spray-drying involves the atomisation of a feed material (a liquid, solution or suspension) and converting this feed material into small droplets in the form of a powder. Spray-driers consist of 6 main components: a feed delivery system, an atomiser, a heated air supply, a drying chamber, a solid-gas separator and a production collection system. Adjustment of the experimental conditions e.g. rate of feed delivered to the atomiser, the inlet air temperature and the flow rate for spray-drying play an essential role in determining the properties of the spray-dried product. Spray-drying may cause polymorphic changes, solvate formation, and the production of amorphous solid or glassy form (Langrish, 2009). Upon spray-drying, the feed solution is atomised into liquid droplets that very quickly form into the solid

particles without allowing the molecules time to crystallise, thus resulting in the formation of an amorphous material (Langrish, 2009).

Spray-drying is a versatile technique that exhibits several benefits like high precision control over particle size, bulk density and degree of crystallinity, in addition to the rapid drying of temperature sensitive materials. However, the central concern about the application of spray-dryers is that it might cause the thermal degradation of the materials especially when operated at high inlet temperatures. Thermal degradation can be overcome by operating lower temperatures and increasing residence times via larger chamber sizes (Chiou *et al.*, 2008).

Some difficulties have been presented, in literature, when spray-drying sucrose (Bhandari *et al.*, 1997). As sucrose has a very low glass transition (50 - 75 ºC), the inlet and outlet temperatures should be carefully controlled so that the outlet temperature does not exceed the T_g of sucrose. No amorphous product will be produced if this condition is not maintained. Therefore, controlling the outlet temperature is crucial for the success of sucrose spray-drying (Bhandari *et al.*, 1997). Other issues in spray-drying involve the need for large amounts of feed solution, low yield levels, safety hazards and the lack of close control over outlet temperatures.

1.7.3 Freeze-drying (lyophilisation)

Freeze-drying is widely used in both pharmaceutical and food industry. Freeze-drying is an important drying method which ensures the gentle dehydration of heat sensitive materials. The process involved consists of three main stages: freezing, primary drying and secondary drying (Oetjen, 2008; Franks, 2008; Rey *et al.*, 2004). The mechanism of freezing is a separation within the system which involves the formation of ice crystals; thus water is removed from the solution as ice, resulting in a freeze-concentrated solute phase. The freezing stage leads to the formation of the amorphous form of the material. This can be accounted for by the increase of the viscosity of solution at low temperatures, which delays the ice formation causing the formation of the glassy state before all water is removed from the solution as ice. Therefore, the

cooling rate of the freezing step is important as it controls the ice crystal size. Furthermore, the sample should be left for an adequate length of time to ensure that the sample is completely frozen.

Primary drying is the step of pulling a vacuum to remove the water from the system by sublimation. The primary drying process usually generates a dry, structurally intact product (Oetjen, 2008; Franks, 2008; Rey *et al.*, 2004). During the sublimation stage of freeze-drying (removal of ice from the freeze- concentrated material), the temperature and pressure are considered as critical parameters to maintain the amorphous structure of freeze-concentrated solids formed in the pre-freezing stage. Once all the ice has sublimed, secondary drying may begin; whereby the small amount of non-freezing bound moisture is removed.

All steps in the freeze-drying process should be performed at temperatures below the glass transition temperature T_g of the material. Freeze-drying produces a non-freezing amorphous network which contains a mixture of the solid amorphous sample and the non-freezing water. This glass tends to have a very low T_g because of the relatively high content of plasticising water. If the system is dried above T_g, the matrix is likely to soften and collapse.

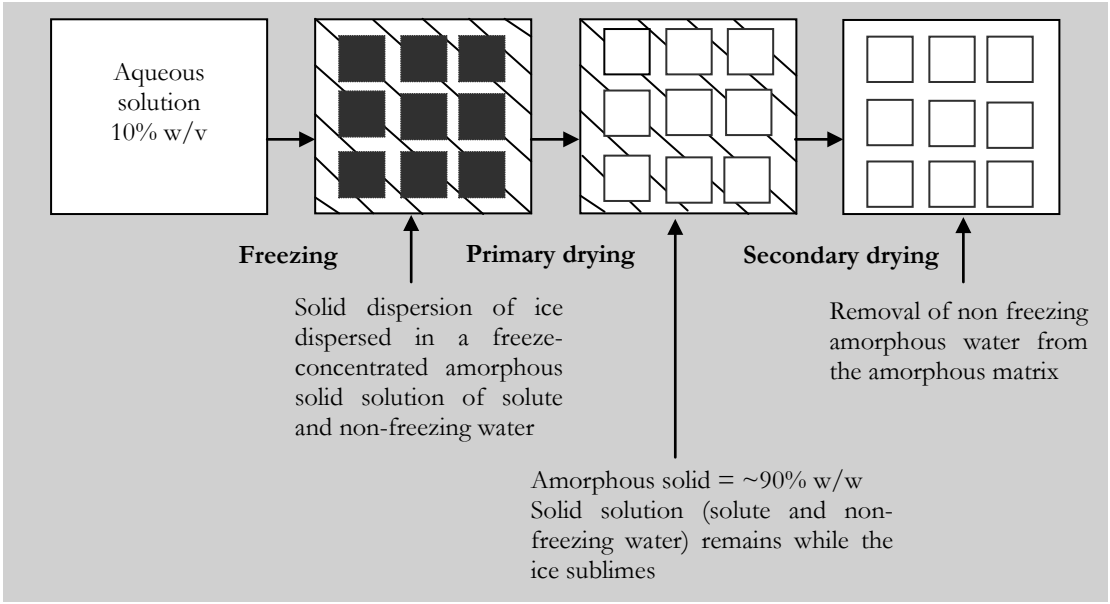

Figure 1.7 A schematic diagram showing the stages of the amorphous state production in freeze-drying.

State diagrams are usually used to derive the optimum freeze-drying parameters and storage conditions. For example, Roos (1997) reported the state diagram of sucrose showing the glass transition curve T_g', T_m' and equilibrium melting temperature T_m (Figure 1.8).

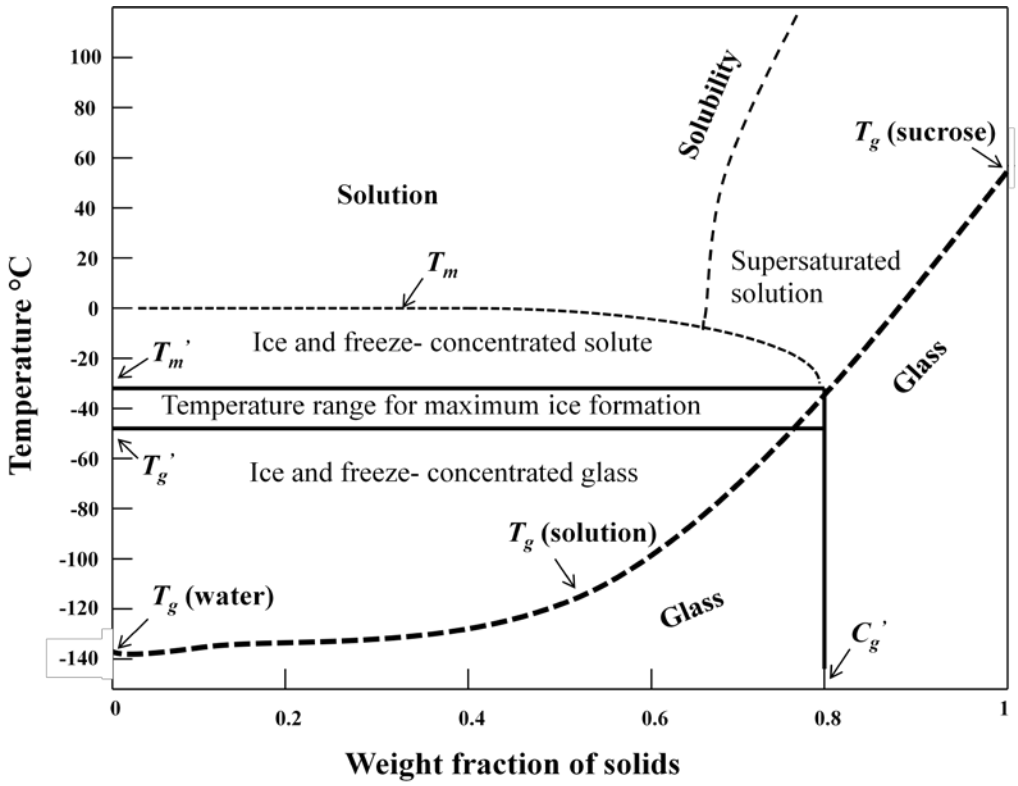

Figure 1.8 State diagram of sucrose showing equilibrium ice melting temperature T_m, onset temperature for ice melting within the maximally freeze-concentrated solute matrix T_m', glass transition of the maximally freeze-concentrated solute matrix T_g' with concentration C_g' and glass transition temperature T_g as a function of the solute concentration (modified from Roos, 1997).

The stability of the freeze-dried products is affected by several factors, most importantly moisture. Freeze-dried products are usually hygroscopic and exposure to moisture during storage can destabilise the product and may lead to re-crystallisation (Haque *et al.*, 2006). For example, freeze-dried sucrose or lactose when opened in air will absorb water from the atmosphere, resulting in lowering the glass transition leading to potential devitrification, collapse and re-crystallisation.

The main advantages of freeze-drying stem from the possibility of storing freeze-dried products at ambient temperature, completeness of rehydration, production of accurate and clean dosing into final product containers, production of a porous and friable structure and minimum damage and loss of activity in delicate heat-liable materials (Mellor, 1978; Faldt *et al.*, 1996; Jacquot *et al.*, 2002).The main disadvantages of freeze-drying on the other hand include long process time, high capital cost of equipment and high energy costs.

Lactose and sucrose have been successfully freeze-dried as reported in literature (Chen *et al.*, 2001; Roos, 1997). Roos (1997) reported the freeze-drying parameters for sucrose and lactose as listed in table 1.6. These experimental conditions will be employed for the production of amorphous lactose and sucrose. This implies that the freezing temperature of both sugars will be kept below T_g' (which is -41 °C for lactose and -46 °C for sucrose) and the freeze- drying process will take place at temperatures below the T_g of the sugar (101 °C for lactose and 62 °C for sucrose). The produced amorphous sugar standards will then be utilized in the investigation of the crystallisation of chocolate sugars and the impact of other factors like minerals and moisture, during crumb manufacture.

Table 1.6 Freeze-drying parameters for sucrose and lactose as reported by Roos, 1997.

	T_g (°C)	T_g' (°C)	T_m' (°C)	C_g' (%w/w)
Lactose	101	-41	-30	81.3
Sucrose	62	-46	-34	81.7

1.7.4 Milling/micronization

Milling can reduce the particle size of the material yet for the production of smaller particle size, micronization is used instead. Milling produces enhanced surface properties that can render crystalline materials fully or partially amorphous (Figure 1.9) (Lefort *et al.*, 2004; Aulton, 2002).

Milled materials may suffer agglomeration of the milled powder and/or reduced stability. This is caused by sample amorphisation and crystalline-to-amorphous conversion. It has to be conceded that reservations have been documented on the relative insensitivity of some analytical techniques to detect small crystallinity changes. Such issues have prompted a considerable interest in detecting amorphous solids at low levels (Lappalainen *et al.*, 2006; Mackin *et al.*, 2002).

As both β- and α- anomers of lactose have different physical properties, the anomeric purity or the β/α ratio of the amorphous lactose should be monitored during chocolate manufacturing process. This implies that milling can be of a great importance to the chocolate manufacturing process as it can produce different β/α content. Willart *et al.* (2007) reported that lactose glass which is obtained by ball milling is in a pure α-anomeric form. As a result, it differs structurally from amorphous lactose obtained by freeze-drying or spray-drying which all produce amorphous mixtures of α- and β- lactose (Jawad *et al.*, 2012).

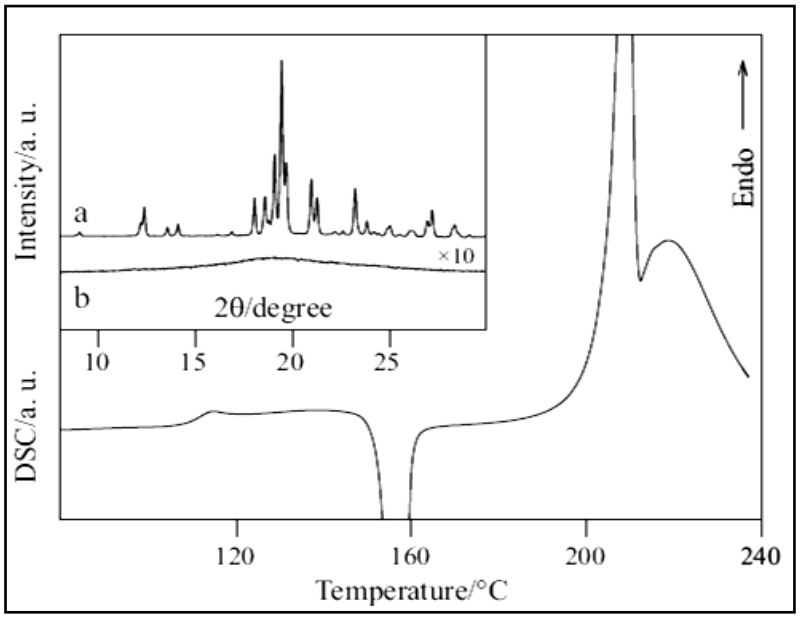

Figure 1.9 DSC heating curves (5°C min^{-1}) of lactose after 30 h of milling treatment. The inset shows X-ray diffraction patterns of lactose recorded at room temperature a – before milling and b – after the 30 h milling treatment (Willart *et al.*, 2007).

Milling was successfully used for the production of amorphous lactose as reported by Willart *et al.* (2007) (Figure 1.9). However, the recorded time for milling was 30 h which is a considerably long time for producing a reasonably small amount of amorphous lactose (unlike freeze-drying which takes long time yet it produces a high number of amorphous lactose batches). Therefore, milling was not considered as the first choice for the production of amorphous sugars.

1.8 Characterisation of the amorphous state

A wide range of techniques have been used to characterise amorphous materials relying on measuring changes in the physical properties of materials including viscosity, density, heat capacity and X-ray diffraction (Lehto *et al.*, 2006).

The selection of an analytical technique for the characterisation of amorphous and crystalline materials is based on several parameters, most notably a high sensitivity and selectivity of the technique especially when studying amorphous/crystalline mixtures. Moreover, the technique of choice will also have to take into account the material that is being analysed (Shah *et al.*, 2006; Lehto *et al.*, 2006) as the levels of detection and quantification depend on the material under investigation rather than the technique itself. For example, the size of the re-crystallisation peak of an amorphous sample is controlled by the molecular structure of the material under investigation rather than the method used for measurement. Therefore, the techniques and methods used in the thesis will all be designed and developed for the characterisation of amorphous sucrose and lactose.

Thermal analytical techniques including differential scanning calorimetry (DSC), differential thermal analysis (DTA), solution calorimetry (SolCal) and isothermal microcalorimetry (IMC) are extensively used in the field of pharmaceutical industry, material science and food science for detecting and measuring amorphous materials (Shah *et al.*, 2006; Lehto *et al.*, 2006; Katainen *et al.*, 2005; Kedward *et al.*, 1998; Ahmed *et al.*, 1996; Itoh *et al.*, 1977).

In addition to the mechanically based methods like dynamic mechanical analysis (DMA), other spectroscopic techniques e.g. nuclear magnetic resonance (NMR), near infrared and FT-Raman spectroscopy, water sorption techniques like dynamic vapour sorption (DVS) have also been used (Jawad *et al.*, 2012; Shrestha *et al.*, 2007; Royall *et al.*, 2005; Katainen *et al.*, 2005; Willart *et al.*, 2004; Mathlouthi *et al.*, 2003; Gustafsson *et al.*, 1998).

1.8.1 Powder X-ray diffraction (PXRD)

X-ray diffraction is a common technique used to study the fingerprint characterisation of crystalline materials and the determination of their structure. Each crystalline solid has its unique characteristic X-ray powder pattern which can be used as a "fingerprint" for its identification. X-rays are diffracted by each material differently, depending on what atoms make up the crystal lattice and how these atoms are arranged (Saleki-Gerhardt *et al.*, 1994; Sebhatu *et al.*, 1994; Shah *et al.*, 2006).

X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. These X-rays are generated within a sealed tube under vacuum, filtered to produce monochromatic radiation, collimated to a concentrate, and directed toward the sample. The interaction of the generated X-rays with the sample produces constructive interference (and a diffracted ray). X-ray diffractometers consist of three basic elements: an X-ray tube, a sample holder, and an X-ray detector.

Diffraction techniques only detect molecular order, thus the disorder is only implied by the absence of the order. Amorphous materials exhibit irregular diffraction of electromagnetic radiation when compared to the crystalline form which exhibits strong diffraction peaks. This is due to the disordered molecular structure of amorphous state (Shah *et al.*, 2006). Standard PXRD has been widely applied to detect amorphism of powders at a detection limit of 5 - 10% w/w (Saleki-Gerhardt *et al.*, 1994; Sebhatu *et al.*, 1994; Yu, 2001).

Chen and colleagues (2001) illustrated that parallel beam PXRD is useful for the quantification of the residual amount of amorphous content in pharmaceutical solid using lactose as the model system. They evidenced that parallel beam PXRD can provide accurate analysis of relatively small amounts (as low as 0.37% w/w) of amorphous content in pharmaceuticals. Haque *et al.* (2005) demonstrated the crystallisation of freeze-dried and spray-dried amorphous lactose by PXRD via the increasing peak intensities in the PXRD patterns.

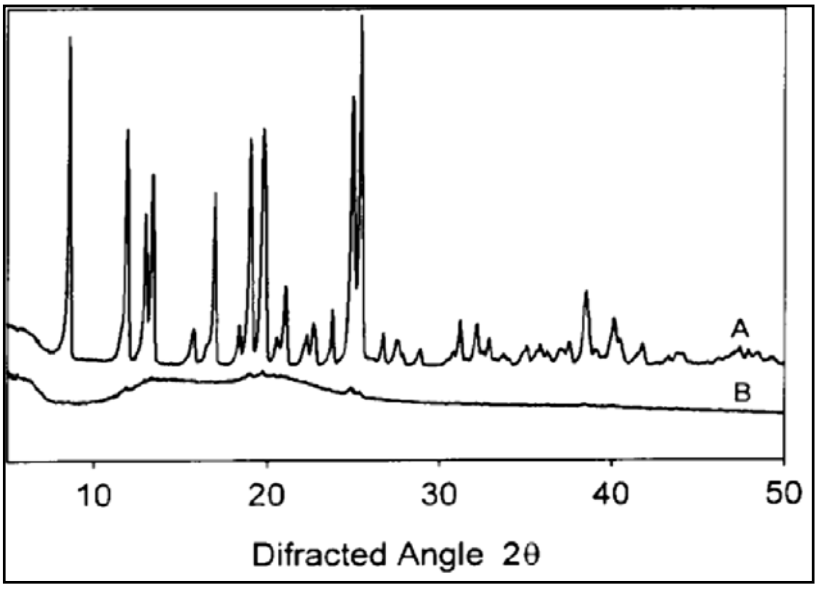

Figure 1.10 X-ray diffraction patterns of sucrose (A) crystalline sucrose and (B) amorphous sucrose (Gloria *et al.*, 2001).

PXRD has several advantages which make it a useful method for amorphous analysis. It is considered a powerful, non-destructive and fast (< 20 min) identification technique. In addition to the minimal sample preparation involved, data interpretation is relatively straight forward.

Nevertheless, PXRD has its limiting shortcomings which include the fact that it requires a homogeneous and single phase material for the identification of unknown materials. Consequently, a heterogeneous or un-evenly distributed sample might generate inaccurate results. Moreover, PXRD requires tenths of a gram of the material which must be uniformly ground into a powder. This is considered a drawback as it might affect the stability of

amorphous powder. Peak overlay might occasionally occur which is particularly noticeable for high angle reflections.

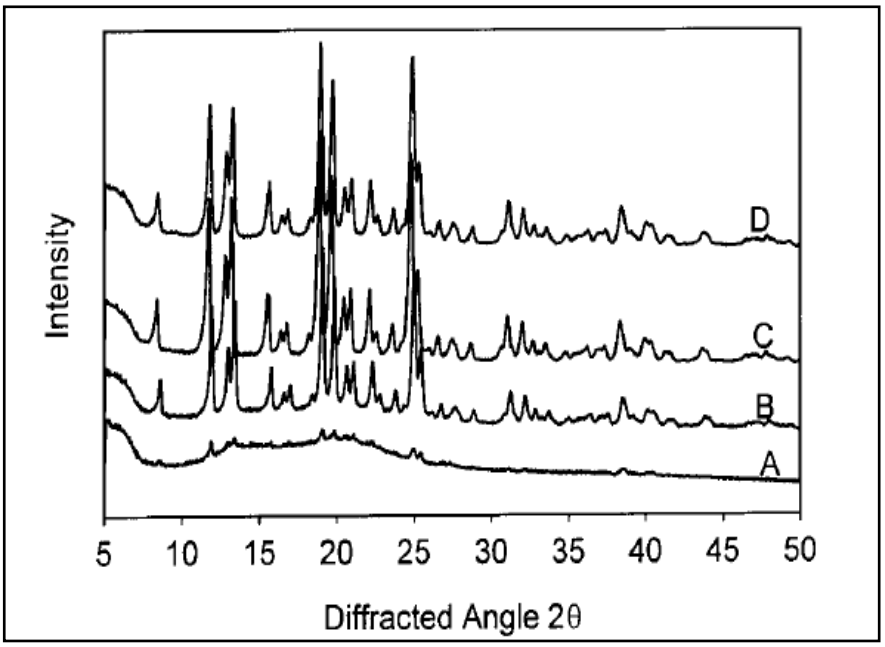

Figure 1.11 X-ray diffraction patterns of dark chocolate samples made with 100% amorphous sucrose. A, after 5 min of mixing; B, after 10 min of mixing; C, after refining; and D, chocolate tablet (Gloria *et al.*, 2001).

Gloria and colleagues (2001) demonstrated that X-ray diffraction is of great benefit when applied to chocolate samples. Measurements of chocolate samples were made with 100% amorphous sucrose. After 5 min mixing of the chocolate mass, sucrose was still in an amorphous state. After 10 min of mixing, sucrose crystallisation appeared to be complete and sharp X-ray diffraction profiles evidenced the presence of crystalline sucrose after refining and conching, and in the final chocolate product (Figure 1.11).

1.8.2 Thermogravimetric analysis (TGA)

TGA is a thermo-analytical technique that determines changes in weight with relation to temperature changes. TGA records variations in mass from dehydration, decomposition and oxidation of a sample. It is principally applied to investigate the thermal stability of a material by monitoring the weight change which occurs as the sample is heated. TGA experiments are

usually conducted in air or in an inert atmosphere, such as helium or argon. Samples size ranges approximately between 2 and 10 mg.

Thermogravimetric analysis is a simple, complete, accurate and responsive method of moisture determination. To obtain accurate and reproducible results, the surface area of the sample should be maximized (Yu, 2001). Besides the long run time, limitations of this technique include difficult method development (numerous scans may be required to optimise run conditions).

Eggleston *et al.* (1996) studied the thermal degradation of crystalline sucrose using DSC and TGA. The analysis was carried out at a heating rate of 15 °C/min from 50 to 500 °C. The DSC scan of crystalline sucrose showed a sharp melting endotherm at 188 °C followed by an endotherm at 229 °C and a broad exotherm at 333 °C. In the TGA thermogram, no weight loss of sucrose was observed during the melting endotherm. This was followed by two subsequent weight loss peaks occurring at 237 °C and 285 °C which correspond to the second DSC endotherm and the onset of the DSC exotherm respectively.

This project will utilise TGA to determine the loss of the adsorbed water of the amorphous sucrose and lactose. In general terms, controlling the moisture content of amorphous sucrose and lactose is essential to hinder any re-crystallisation upon storage. TGA is a very useful tool for monitoring the moisture content of amorphous sugars after both freeze- or spray-drying and upon storage.

1.8.3 Differential scanning calorimetry (DSC)

Conventional differential scanning calorimetry (DSC) is widely used in pharmaceutical industry for characterising and quantitating mixtures of polymorphs and amorphous solids. DSC has become a principal technique for the determination of T_g especially in pharmaceuticals (Lehto *et al.*, 2006; Kedward *et al.*, 1998; Kerc *et al.*, 1995). It has been extensively used in the characterisation of phase transitions, such as glass transitions, melting points, oxidative/thermal

stability and exothermic decompositions (Islam *et al.*, 2010; Liu *et al.*, 2007; Lehto *et al.*, 2006; Foubert *et al.*, 2003; Roos *et al.*, 1990).

DSC is a thermo-analytical technique based on heating (or cooling) a sample typically under a linear rate and measuring heat absorbed or evolved as temperature increases. DSC exhibits a very high sensitivity in detecting the energy changes or heat capacity changes involved in phase transitions (Giron, 1986).

In DSC analysis, amorphous components may sometimes re-crystallise during the heating process to form a more stable form of the compound, thus producing an exothermic signal on the DSC scan. A typical DSC scan for an amorphous drug phase will show two peaks: a lower temperature exotherm, reflecting conversion to the crystalline phase, followed by the melting endotherm. A small endothermic transition may be evident prior to the exotherm reflecting the glass transition (Figure 1.12) (Gloria *et al.*, 2001; Corrigan, 1995).

DSC offers several advantages which include the use of a relatively small sample size. Moreover, samples can be crimped which provide shielding from any external effect from the atmosphere. Furthermore, sample preparation is not complicated and the time needed for analysis is less than one hour as the scans can range from 2°C/min to 10°C/min (McGregor *et al.*, 2003).

DSC has specific advantages for its application to analyse amorphous sucrose and lactose. One is that both amorphous sucrose and lactose are fragile glass formers and so will have a relatively significant step change in the signal for the glass transition (Haque *et al.*, 2006; Simperler *et al.*, 2006). Furthermore, both amorphous sugars have a tendency to re-crystallise after the glass transition (Islam *et al.*, 2010; Gloria *et al.*, 2001). Thus, both advantages may be used to judge whether the sugars are amorphous. In addition, crystalline α-lactose monohydrate has a hydrate loss peak in the DSC thermogram which is absent for the amorphous forms. Therefore, this peak may be used as a defining parameter to indicate the absence of crystallinity (Islam *et al.*, 2010). However, the disadvantage of DSC for amorphous sucrose and lactose is

that the melting peak of both sugars has a contribution from degradation which complicates the signals (Lee *et al.*, 2011).

Based on the investigations carried out by Gloria and colleagues (2001) to compare amorphous and crystalline sucrose using DSC, crystalline sucrose exhibits a single melting peak at 188°C (Figure 1.12). However, amorphous sucrose shows T_g at 57 °C, a crystallization peak at 105 °C and an onset melting temperature of 176 °C.

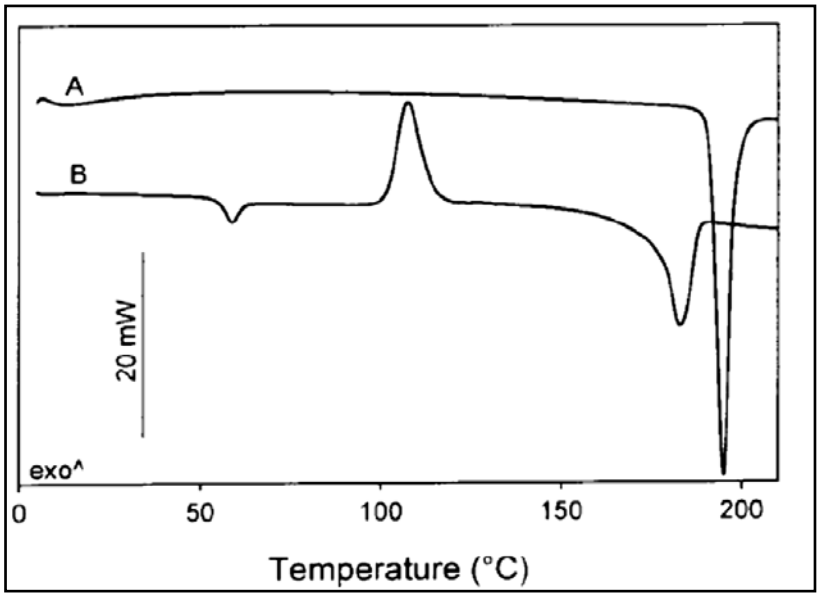

Figure 1.12 DSC scans of sucrose (A) crystalline sucrose and (B) amorphous sucrose (Gloria *et al.*, 2001).

Saleki-Gerhardt *et al.* (1994) applied DSC to measure the enthalpy changes associated with crystallisation of amorphous sucrose. The limit of detection (LoD) of DSC with respect to amorphous sucrose was determined as 1% w/w. Gloria and co-workers (2001) also applied DSC to study the changes in the physical state of sucrose during dark chocolate manufacture. Three batches of dark chocolate were manufactured. Batch 1 contained 100% crystalline sucrose, batch 2 contained 50% crystalline sucrose and 50% amorphous sucrose and batch 3 contained 100% amorphous sucrose. DSC scans of dark chocolate samples containing amorphous sucrose were characterised by a glass transition at 63 °C, a sucrose crystallisation peak at 105 °C and a melting peak at 188 °C. Chocolate prepared with 100% amorphous sucrose showed a fast reduction in

the enthalpy of the crystallisation peak at 105 °C during the mixing process. The amount of amorphous sucrose present after 5 mins of mixing was 49.0% w/w whereas that present after 10 mins of mixing was 7.4% w/w. Gloria *et al.* (2001) concluded that when amorphous sucrose was used for dark chocolate processing, it had completely crystallised in the early stage of the processing i.e. mixing and was not present in the final dark chocolate product. This is mainly due to the sucrose crystals which act as nucleation sites favouring the crystallisation process. Although the majority of amorphous material may re-crystallise, a small fraction may still persist.

1.8.4 Dynamic mechanical analysis (DMA)

Dynamic mechanical analysis (DMA) is a technique which is based on applying an oscillating force to a sample of known geometry and analysing the materials response to that force. The force is transmitted to the sample via the drive shaft. The applied force causes a sinusoidal stress to the sample which generates a deformation or a sinusoidal strain. The ratio of stress to strain constitutes an important parameter to measure by DMA which is the modulus. Therefore, this mechanical operation provides information about the damping or the deformation of the sample (tan delta), the stiffness (modulus) from the sample recovery and the flowing properties (viscosity) of the sample from the phase lag (Royall *et al.*, 2005).

In DMA, to improve the reproducibility of the observed mechanical data, a number of technical steps should be taken into consideration such as a properly calibrated instrument (temperature and force), a well prepared specimen (even thickness, parallel sides and right angle), selecting the right geometry, and applying both reasonable strain and heating rates (PerkinElmer, DMA manual).

DMA is used to determine the glass transition T_g of a sample by heating it under an oscillating load. This process involves the construction of a logarithmic plot of the modulus against a linear temperature scale where the glass transition is characterised as a large drop in the storage modulus. Moreover, a concurrent peak in the tan delta is observed.

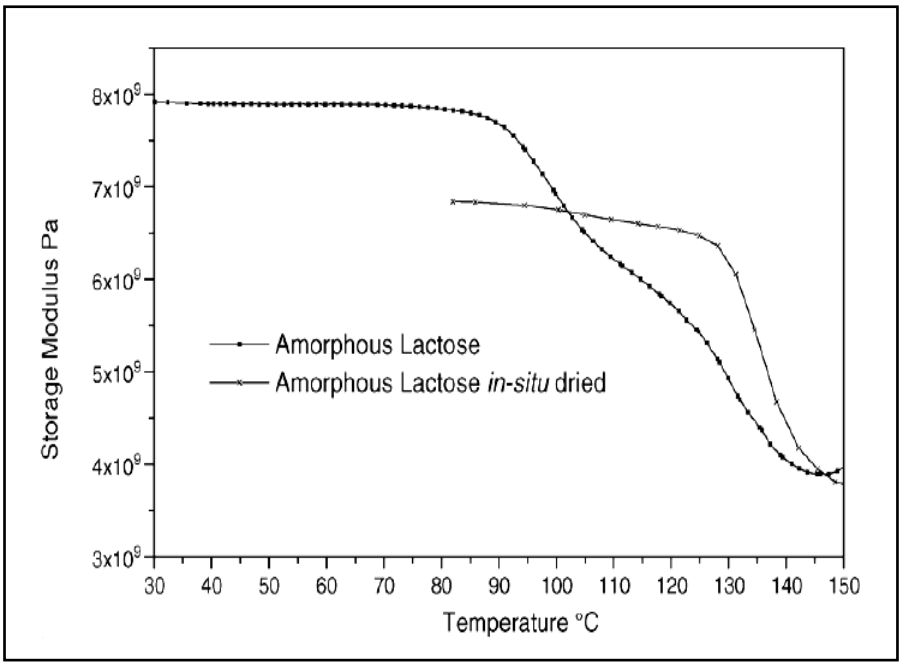

Figure 1.13 A typical powder-pocket DMA response for amorphous lactose showing a comparison between the modulus signals of spray-dried amorphous lactose, and in situ dried amorphous lactose (in situ drying consisted of heating to 110 °C then cooling to 80 °C and then re-heating to 250 °C), all with a temperature scan rate of 5 °C/min (Royall *et al.*, 2005).

The advantages of DMA lie in its capability to detect thermal events such as glass transition and related phenomena. DMA is also highly useful for measuring the viscoelastic/rheological properties of a wide range of samples. However, sample preparation, interpretation of results and determining the optimal measuring conditions can be considered as the main disadvantages of this technique. Variations in the sample preparation procedure could lead to poorly reproducible and inaccurate results (Craig *et al.*, 1995). Furthermore, interpretation of the results is not simple when it comes to complex sample matrices or multi-component systems.

The limit of detection of lactose for DMA was determined to be 2.8

% w/w amorphous content while the limit of quantitation was 9.4% for the powder-pocket DMA (Royall *et al.*, 2005). DMA will be used in this project for the characterisation of amorphous lactose, sucrose and lactose/sucrose mixtures.

1.9 Determination of the chemical purity of lactose and sucrose

Nuclear magnetic resonance (NMR) and polarimetry will be employed to determine the chemical purity of lactose and sucrose. The purity of lactose involves the anomeric ratio of both β- and α- anomers of lactose resulting from the mutarotation phenomena discussed earlier in this chapter; whereas, the purity of sucrose involves the inversion reaction into its invert sugars glucose and fructose.

Based on literature search, NMR was applied to determine the anomeric content of lactose (Willart *et al.*, 2004). NMR was also able to characterise amorphous lactose (Gustafsson *et al.*, 1998). However, in the scope of this thesis, NMR will be applied to determine the purity of both crystalline and amorphous lactose rather than characterising the amorphous form. Polarimetry was extensively used in literature to measure the optical rotation of chiral compounds including sucrose.

1.9.1 Nuclear magnetic resonance (NMR)

NMR is a spectroscopic technique that exploits the magnetic properties of nuclei possessing a spin quantum number of ½ (for proton NMR) as they are brought into an external magnetic field B_o (also referred to as H_o). The magnetic momentums of the nuclei become oriented in one of two directions with respect to the field depending on its magnetic quantum state (Bugay, 2001). The energy required for the spin-flip depends on the magnetic field strength at the nucleus (Figure 1.14). In the absence of any applied field, there is no energy difference between the spin states. However, applying a magnetic field H_o causes the separation of energies of the spin states and consequently the alignment of nuclei with the applied field.

NMR spectroscopy applies radiofrequency radiation to induce transitions between different nuclear spin states of samples in a magnetic field and thus obtain the NMR spectrum.

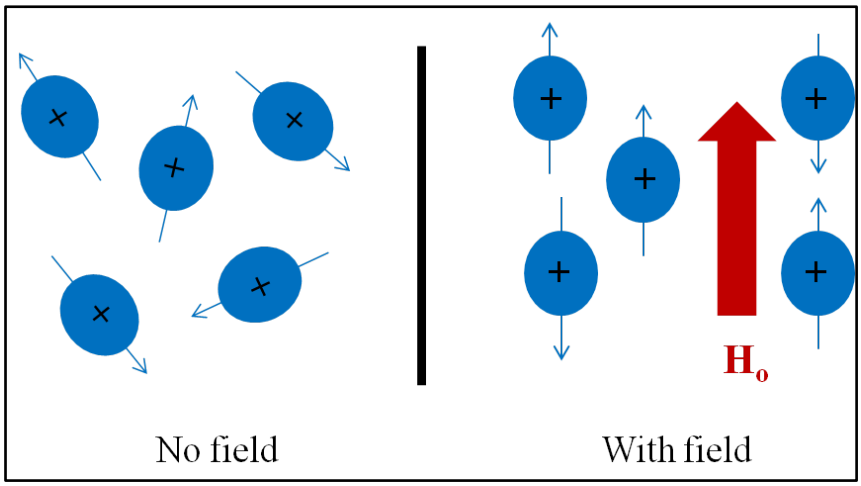

Figure 1.14 The orientation of nuclei in the absence and the presence of an external magnetic field.

Energy levels are caused by the principal, so-called Zeeman interactions Hz - between the magnetic moment of the nucleus and the surrounding magnetic field B_o. Another interaction of interest is the chemical shift. This chemical phenomenon results from the variation between the opposing field and the effective field (the magnetic field around the nucleus). Dipole-dipole H_d is another type of interaction which is described as the direct magnetic coupling of two nuclei through space. The chemical shift H_{cs} is an essential parameter and interaction in NMR. H_{cs} provides the most diagnostic information of the measured NMR spectrum (Bugay, 2001; Tishmack *et al.*, 2003).

NMR is technique of high specificity and which provides information about the structure of the material as well as any existing contaminants. Gustafsson *et al.*, (1998) reported that the advantage of employing NMR to study the structure or crystallisation of sugars revolves around its ability to provide a non-destructive and highly sensitive approach that could detect as low as 0.5% amorphous lactose in a mixture with high reproducibility (Figure 1.15).

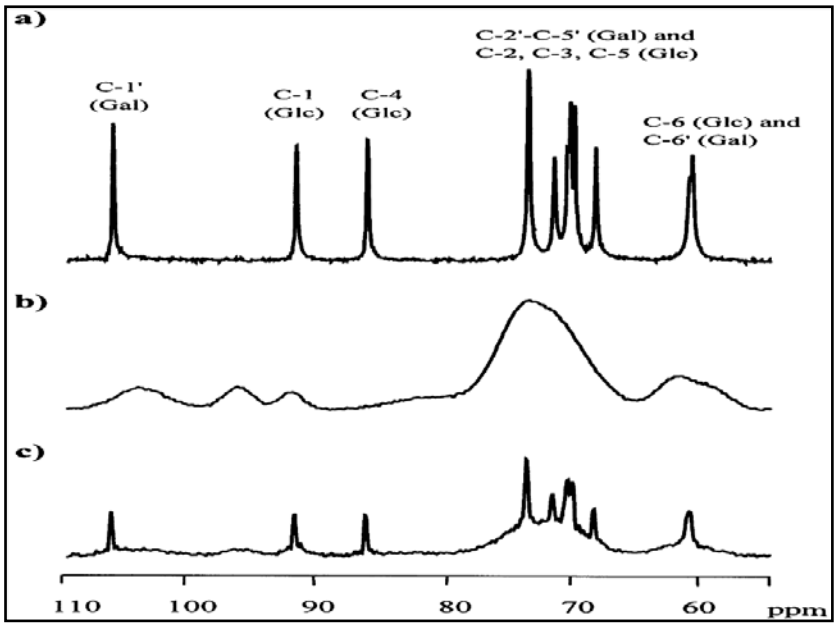

Figure 1.15 A ^{13}C-NMR spectrum of lactose (a) 100% crystalline lactose sample; (b) 100% amorphous lactose sample; (c) 90% crystalline–10% amorphous lactose (Gustafsson *et al.*, 1998).

^{1}H-NMR has been increasingly applied to investigate the mutarotation of α- and β-anomers of lactose. It was elucidated that ^{1}H-NMR was able to differentiate between both anomers by exhibiting two characteristic NMR signals of the α- and the β- lactose at 6.3 and 6.6 ppm respectively (Willart *et al.*, 2004; Jawad *et al.*, 2012).

1.9.2 Polarimetry

Polarimetry is an analytical technique which can measure the tendency of molecules to rotate a plane of polarised light and determine their concentration in solution. The mechanism by which the instrument works is that non-polarised light (coming from a light source) passes through a polarising filter where it becomes polarised. The polarised light then passes through the sample (Kar, 2007; Anslyn *et al.*, 2006; Gergely, 1989). If the sample in the cell is optically active, a change in the intensity of illumination is observed indicating the rotation of the plane of polarised light. The analyser then turns to its original illumination where the angle is measured in degrees. Molecules which rotate the plane of polarised light to the right (or clockwise) will

produce a positive (+) specific rotation $[\alpha]_D$, while molecules which rotate the plane of polarised light to the left, will have a negative (-) specific rotation $[\alpha]_D$ (Gergely, 1989).

The observed optical rotation depends on many variables such as the nature of the sample, the concentration of the optical active components of the sample, the pathlength of the flow cell, the wavelength of the light source and the temperature of the sample.

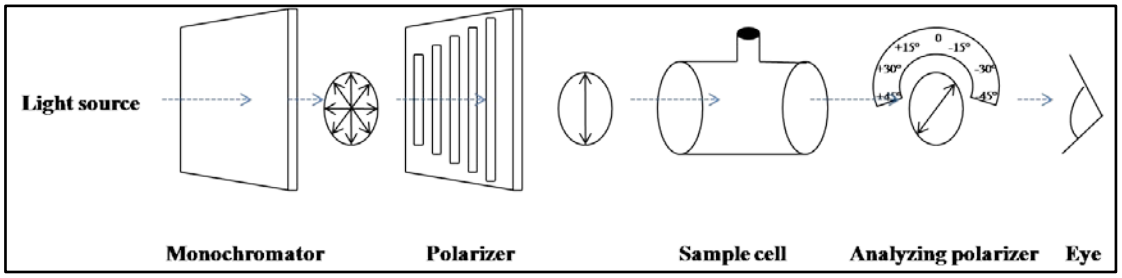

Figure 1.16 A schematic diagram of a polarimeter.

The specific rotation $[\alpha]_D$ is calculated as follows:

$$[\alpha]_D = \frac{\quad}{\quad} \qquad \text{Equation 1}$$

Where $[\alpha]_D$ is the specific rotation in degrees at a wavelength of sodium lamp light source λ= 589 nm, OR is the observed optical rotation in degrees, l is the pathlength in dm and C is the concentration in % (g/100 mL). For example, the $[\alpha]_D$ of 1 g/100 mL sucrose solution at 20 °C has been reported as + 66.5° (Mathlouthi *et al.*, 1995).

1.10 Project rationale

The crystallisation of fats in chocolate, as discussed in the aforementioned section, has been widely covered in literature (Ziegleder *et al.*, 1990; Loisel *et al.*, 1998; Tietz *et al.*, 2000; Sato, 2001; Attaie *et al.*, 2003; Foubert *et al.*, 2004; Vereecken *et al.*, 2007). However, fats are not the only source of crystallisation in chocolate. Sugars (which constitute up to 50 % w/w of the chocolate composition) also play a vital role in re-crystallisation affecting both the flavour and the quality of chocolate.

Amorphous sugars can be introduced to chocolate in a number of different ways. The milk powder (containing lactose) used in the production of the chocolate is commonly produced by spray-drying leading to the formation of amorphous lactose. The glassy state of lactose is capable of retaining some of the milk fat making it unavailable to help the chocolate flow (Beckett, 2000). Amorphous sugars may also be introduced to chocolate during the crumb manufacturing process. Crumb is made up with liquid milk yet amorphous lactose may be introduced during processing. During crumb production, the concentrated milk, sugar and cocoa liquor mixture undergo drying under reduced pressure to form a solid material. The different ingredients including sugars undergo a milling step which may contribute to the introduction of amorphous content into the crumb.

The impact of the presence of amorphous sugars in the chocolate crumb can vary from batch to batch depending on the quantity of the amorphous sugars present in the mixture. The higher the amount of amorphous sugar in chocolate, the higher its water content and aroma retention will be (Beckett, 2000). The large specific area of amorphous sucrose and lactose might lead to the adsorption of large amounts of volatiles causing variation in the chocolate flavour. Moreover, amorphous sugars are highly hygroscopic therefore they are capable of absorbing water from the environment and other chocolate ingredients, creating not only potential difficulties during processing and storage but also flavour variations as chocolates with high water content usually exhibit an unacceptable gummy texture in the mouth (Minifie, 1979). Small amounts of absorbed water may affect the rheological and flowability of the chocolate product. Furthermore, as sucrose constitutes up to 50% w/w of the chocolate composition, the closely related particles can adhere together due to water uptake by the glassy state of sucrose. Sugar aggregates can build up as a result which tightly hold together even if fat melts which severely affects the flowing properties of the chocolate crumb (Beckett, 2000). Amorphous sugars may also undergo a re-crystallisation process leading to a decrease in viscosity and an increase in water activity which may cause chocolate degradation. Therefore, a key issue is the controlled

monitoring of the amorphous content in chocolate as the uncontrolled introduction of small amounts of amorphous sugar into the chocolate can change the mouth feel of the chocolate varying from batch to batch leading to both a poor chocolate quality and customer dissatisfaction. The first step in controlling the potential generation of amorphous sugars and its impact on customer satisfaction is to develop and validate methods for generating controlled samples of amorphous lactose and sucrose and then detecting their crystallisation within a model chocolate system. Therefore, in order to establish and evaluate an analytical approach that will fulfill this need, the research reported within this thesis addresses the following aim and objectives.

1.11 Aims and objectives

The aim of this project is to investigate the phase changes of sucrose and lactose within a suitable model. Moreover, this project aims at understanding the effect of the recipe and process variables upon the mechanisms and nature of crystal formation during chocolate manufacture.

The objectives of the project are:

Objective 1: Characterisation of the crystalline forms of lactose and sucrose and establishment of the properties of the feed material within the model system.

Objective 2: Preparation and characterisation of amorphous sucrose and lactose standards. Production of amorphous sucrose and lactose will be performed by spray-drying and freeze-drying. Assay development including a range of thermal and spectroscopy techniques which will be employed to characterise the samples.

Objective 3: Determination of the chemical and physical purity of both dried sucrose and lactose. This will involve investigating the purity of sucrose with respect to inversion and measuring the β/α anomeric ratio of lactose.

Objective 4: Characterisation of the mutarotation kinetics of lactose in aqueous solution and studying the impact of temperature on the kinetics of the mutarotation process.

Objective 5: Production of model systems containing different controlled levels of sugars and minerals. Measurement of crystallisation: The samples produced in task 2 will be characterised by the techniques selected in tasks 1 & 2. This will allow the measurement of the presence of sugar crystallinity within the chocolate samples and the impact of such variables on the crystallisation of the final recipe.

Chapter two – Production and characterisation of amorphous lactose

The aim of this chapter is to report the development of a robust method for the production of amorphous lactose standards. The produced lactose standards will be subsequently incorporated into model systems resembling chocolate crumb (chapter 5). To allow the characterisation of crystallisation within these models systems, it was important to have a reliable method for the generation of amorphous lactose. The methods that were used to confirm the presence of highly amorphous material are presented in this chapter. However, it was also important to confirm the chemical composition of lactose before and after rendering it amorphous because lactose undergoes mutarotation. Thus, the measurement of the anomeric β/α ratio of lactose in both crystalline and amorphous forms was deemed a fundamental research objective of the work discussed in this chapter.

2.1 Introduction

Lactose constitutes approximately 8% w/w of the chocolate composition (Beckett, 2000). It can influence the flavour and the quality of the chocolate crumb due to its role in the re-crystallisation process of the crumb (Beckett, 2000).

Lactose is a reducing disaccharide which consists of β-D-galactose and α/β-D-glucose fragments bonded through a β1-4 glycosidic linkage. It constitutes approximately 2 - 8% w/w of milk (Fox *et al.*, 1998). As previously illustrated, lactose can exist in two anomeric forms, α-lactose and β-lactose, which differ in the orientation of the hydrogen and the hydroxyl group on carbon atom number 1 (Figure 2.1). The α- and β- anomers of lactose exhibit different physicochemical properties. For example, there is a 7 fold difference between the observed solubility of the crystalline forms of the isomers in water (Table 2.1).

Table 2.1 Physico-chemical properties of crystalline α-lactose monohydrate and crystalline β-lactose (McSweeney *et al.*, 2009; Drapier-Beche, 1999).

Property	**α**	**β**
Melting Pt °C	202	252
$[\alpha]_D$ at 20 °C	84 °	34°
Solubility (in H_2O) 20 °C g/100 mL	7	50
Specific heat	0.299	0.285
Specific gravity 20°C	1.54	1.59
Heat of combustion (kJ/mol)	5687	5946

In aqueous solutions, both α-lactose and β-lactose may undergo mutarotation (Fox *et al.*, 1998) and it is believed that they interconvert following the scheme given in figure 2.1. This mechanism is based on study reports on the mutarotation of related sugars principally glucose (Silva *et al.*, 2006; McMurry *et al.*, 2004). The mutarotation of lactose involves the formation of a free aldehyde form on the glucose unit which is initiated by the protonation of the O5, followed by breakage of the O1-H bond (Silva *et al.*, 2006; McMurry *et al.*, 2004). Over time, a steady state is reached between the two forms of lactose present in solution. At neutral pH and room temperature, the equilibrium amounts in water are approximately 63% β-lactose and 37% α-lactose (Fox *et al.*, 1998). The exact composition and the time taken to reach equilibrium between the two forms are dependent on concentration, pH and temperature. Typical equilibration times are reported to fall within the 3.5 to 6.5 h range. It is generally accepted that the mutarotation of lactose in aqueous solution follows first order kinetics for reversible reactions approaching equilibrium (Haase *et al.*, 1966; Patel *et al.*, 1970; Raghava *et al.*, 2000).

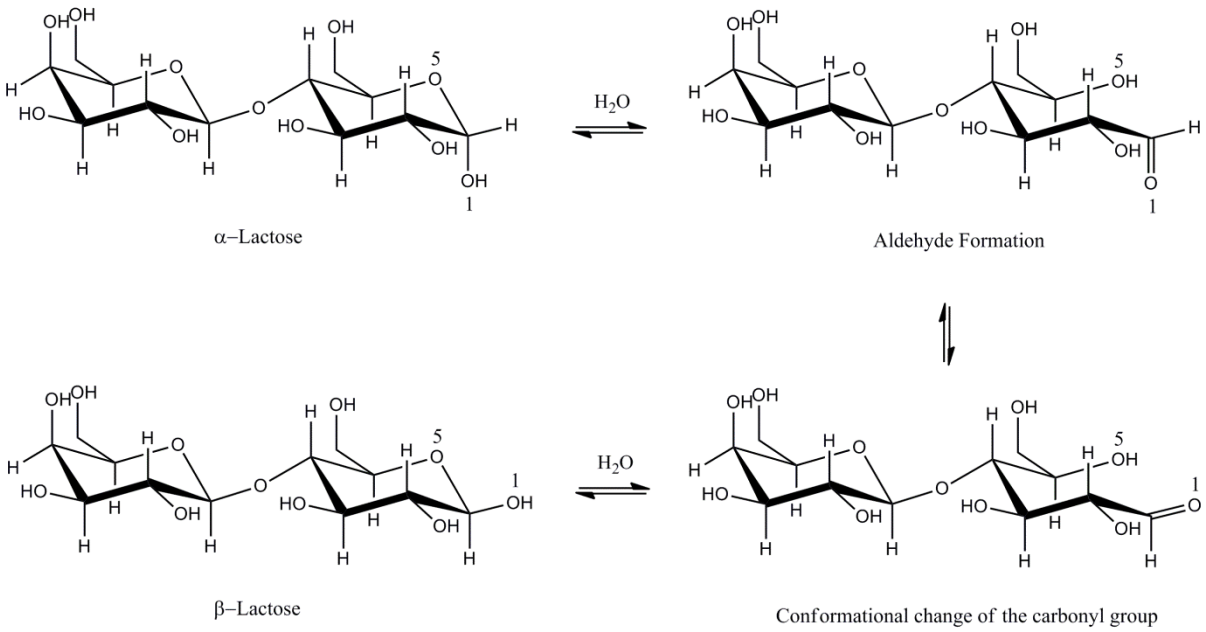

Figure 2.1 A representation of the mutarotation mechanism of lactose based on the mechanism published by Silva *et al.*, 2006 and drawn using Chemdraw®. This is an early model that clearly can not completely describe the equilibrium. The reason for the problem is that the open structure with the carbonyl group can not be observed in the UV as it does not live for a long time. For a discussion of the initial ring opening, the initial model will be adequate. A better model will be proposed in chapter 3.

Amorphous lactose can be introduced to chocolate via different routes. The milk powder (containing lactose) used in the production of chocolate is usually produced by spray-drying leading to the formation of amorphous lactose (Beckett, 2000). The glassy state of lactose is capable of holding some of the milk fat rendering it unavailable to enhance the chocolate flowability. As discussed in the introduction and will be detailed in chapters 4 & 5, the crumb manufacturing process involves exposing the chocolate ingredients (sugar, milk, cocoa liquor) to reduced pressure, high temperature and milling. All of this can introduce amorphous sugars especially amorphous lactose into the crumb.

Therefore, amorphous lactose standards with known and controlled β/α composition should be produced in order to monitor the process of crystallisation within crumb and to take

account of the impact they might have on its crystallisation profile. Knowledge of the anomeric composition is also vital to ensure a better accuracy and sensitivity in the thermo-analytical measurements taken for the amorphous lactose standards, because the different anomers will show different responses. The generated lactose standards will then be incorporated into the model crumbs that will be prepared and tested in chapter 5.

Spray- and freeze-drying are the most commonly employed techniques for the production of amorphous lactose (Ramos *et al.*, 2005; Buckton *et al.*, 2002; Fox *et al.*, 1998; Gustafsson *et al.*, 1998; Hill *et al.*, 1998). Both of these methods use aqueous solutions of lactose as their feed material. However, the anomeric composition of the resulting amorphous solids is rarely described. A survey of pharmaceutical literature over the last 20 years which was conducted by the author demonstrated that of the 103 articles that contain "amorphous lactose" in their title, only 4 articles explicitly reported the ratio of the β/α anomer composition within the amorphous lactose formed. Without measuring the β/α ratio, such studies are incomplete, not least due to the potential differences in the physicochemical properties of the anomers (McSweeney *et al.*, 2009).

Several attempts to investigate the β/α content of amorphous lactose have been reported. Buckton *et al.* (2002) investigated the influence of feed temperature on the anomer composition of spray-dried lactose and observed that between room temperature and 40 °C the amount of the β- anomer was lower than the α- anomer. However, the situation was reversed above 50 °C. The conclusion drawn from this observation was that at low feed temperature, mutarotation occurred during spray-drying. However, at high feed temperatures, the composition of the amorphous material reflected the anomer ratio present in the feed aqueous solution at that storage temperature. The authors reported that the feed solutions were equilibrated at their assigned storage temperatures. However, the equilibration time for mutarotation to occur at room temperature is typically 3.5 – 6.5 h, and as no equilibration times are provided it is not clear whether anomer equilibrium had been achieved at the lower feed

temperatures (Buckton *et al.*, 2002). To add further ambiguity in an earlier study by the same group of authors, it was reported that no β-lactose was present in spray-dried samples produced using similar parameters as the later study (Chidavaenzi *et al.*, 1997).

Roetman and van Schaik produced amorphous lactose employing a wide range of spray-drying conditions (Roetman *et al.*, 1975). The amount of β-lactose measured in these samples was consistently higher than the α-anomer and it was observed that the ratio between the two was controlled by the temperature of the feed solution (before being passed into the spray- drier), the outlet temperature of the spray-drier and later, the storage temperature of the powder. This work indicated that mutarotation occurs in the feed solution, during spray- drying and subsequently in the solid amorphous state. Roetman and van Schaik claimed that there is a universal β/α isomeric ratio of 1.25 (55% β and 45% α), which all amorphous forms of lactose approach and the rate of achieving this solid state equilibrium depends on the storage temperature and water content (Roetman *et al.*, 1975). In the latter study, the feed solutions were held at different temperatures, but the length of the holding time was not described, so it is difficult to judge whether the mutorotation equilibrium had been attained before drying. Furthermore, both polarimetry and gas chromatography (GC) were used to measure the β/α ratio but there were significant differences between the β/α ratios measured by the two different methods (Roetman *et al.*, 1975).

Ramos *et al.*(2005) produced samples of spray-dried lactose using the same feed concentration and similar spray-drying conditions as the Roetman and van Schaik study (Roetman *et al.*, 1975). However, the former study reported the opposite in terms of the dominant anomer, with 34% β and 66% α present in the isolated amorphous lactose samples. It was claimed that the amorphous material had the same composition irrespective of the isomeric composition of the starting material, but neither the feed temperature nor the feed standing time were discussed (Ramos *et al.*, 2005).

A study conducted by Listiohadi *et al.* (2009) characterised freeze-dried amorphous lactose and observed an anomeric content of 59% β and 41% α, a β/α ratio of approximately 1.45. Clearly the postulate of Roetman and van Schaik (Roetman *et al.*, 1975) of a universal anomer composition with a β/α ratio of 1.25 was not confirmed.

Several anecdotal reports (Ramos *et al.*, 2005; Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997) utilised different techniques (polarimetry and a gas chromatographic method based on silylation) to determine the anomer content in lactose samples. The complicated and time-consuming nature of these assays is the likely cause of the paucity of data describing the anomer composition of amorphous lactose. Powder X-ray diffraction (PXRD) can only be applied to crystalline materials and has been used to determine the anomer content in lactose. However, when PXRD is applied for the anomeric characterisation of amorphous lactose such measurements are in fact made post re-crystallisation rather than on the true amorphous form (Haque *et al.*, 2005). The assumption made is that the anomer content in the re-crystallised form reflects the anomer ratio in the amorphous material (Haque *et al.*, 2005). This is unlikely unless water has been completely excluded from the amorphous material and from its storage environment during re-crystallisation. Lactose present in a disordered amorphous solid diffracts X-rays in a diffuse halo, rather than at discrete angles typical of ordered crystalline materials (Shah *et al.*, 2006).

Solid-state nuclear magnetic resonance, ^{13}C-NMR, has also been used to characterise lactose samples (Gustafsson *et al.*, 1998; Willart *et al.*, 2004; Lefort *et al.*, 2006). Gustafsson *et al.* (1998) used peak broadening to identify the presence of amorphous material and reported spray-dried lactose to be 100% amorphous but the authors did not determine the β/α ratio within the amorphous lactose samples. Lefort *et al.* (2006) refined the NMR approach. Their solid state ^{13}C-NMR work identified the fraction of the β- anomer in milled lactose samples which were also approaching 100% amorphous (Lefort *et al.*, 2006). The time taken to conduct these experiments (4 to 6 h) has undoubtedly led to the lack of further published studies using solid-state NMR to

characterise the anomeric content in amorphous lactose. In order to allow NMR to be quickly and routinely applied in the characterisation of amorphous lactose, the development of a conventional solution based NMR approach using dimethyl sulphoxide (DMSO) as a solvent is required. Developing a DMSO method is rational since the mechanism for mutarotation requires the protonation of lactose (Figure 1.1), as DMSO is an aprotic solvent, it can be assumed that mutarotation in DMSO is negligible. Thus the anomeric composition present in a DMSO solution can be assumed to be equivalent to that found in an amorphous sample of lactose before the solution has been prepared. The only report quoting the determination of the anomer ratio by solution based (DMSO) proton NMR makes this assumption (Willart *et al.*, 2004) although this earlier work only describes the application of this technique to crystalline anhydrous α-lactose, a milled and a re-crystallised sample of this material. The fractions of the β-anomer were reported to be <1%, <4% and equalling 50% respectively (Willart *et al.*, 2004). However, no discussion of the technique validation was given and no attempt has been made to apply this technique to spray- or freeze-dried lactose samples. The first study objective for the work presented in this chapter was therefore to validate the application of DMSO proton NMR for the determination of the anomer content of amorphous lactose.

The results from the little work that has reported the anomeric composition of amorphous lactose have been equivocal. Lefort *et al.* (2006) produced predominantly amorphous α-lactose by controlled ball milling of anhydrous α-lactose for 30 h. Upon storage of these samples at elevated temperatures approaching the glass transition of lactose, a solid-state mutarotation to the β-anomer was observed. A final ratio of 50% β and 50% α indicated that the mechanism in the solid-state is different from the mutarotation reaction pathway recorded in aqueous solution (Lefort *et al.*, 2006). A 1:1 ratio of anomers is almost never observed for spray- or freeze-dried amorphous lactose. Furthermore, there is a broad range of anomeric ratios reported (Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997; Buckton *et al.*, 2002; Listiohadi *et al.*, 2009).

Therefore, the wide range of reported anomer content of amorphous lactose is likely to be a result of the limited analytical methods available and a lack of control with respect to the equilibration time of the feed solution before drying. Spray- and freeze-drying are commonly employed in the pharmaceutical and food industries for the bulk manufacture of amorphous lactose (Fox *et al.*, 1998). Therefore, monitoring the anomeric content of spray- and freeze-dried lactose does require re-examination. In order to broaden the number of analytical approaches available and to address the contradictory reports concerning the anomeric content of amorphous lactose in the literature, there is a need to establish a quantitative measurement by ^{1}H-NMR of the α- and β- content in amorphous lactose produced by different drying methods.

The high focus on lactose mutarotation and the ratio of β- and α- anomers in the project arises from three main hypotheses:

1. Mutarotation affects the rate of crystallisation of lactose.
2. Impurities can either inhibit or seed crystal growth.
3. The difference in sweetness anomeric β- and α- forms precipitate.

The first hypothesis is based on the assumption that mutarotation is one of the important factors that affect the crystal growth of lactose (Twieg *et al.*, 1968). Mutarotation affects the rate of crystal growth at temperatures below 30 °C. However, at higher temperatures, the rate of mutarotation significantly increases and thus its impact on the crystal growth diminishes (Brinkman, 1976). Twieg *et al.* (1968) established the term 'isomer balance' to illustrate the impact of mutarotation on crystallisation. The isomer balance denotes the percentages of α-lactose and β-lactose present in solution during crystallisation.

$$\beta\text{-lactose crystals} \rightleftharpoons \beta\text{-lactose} \overset{\text{step 1}}{\rightleftharpoons} \alpha\text{-lactose} \overset{\text{step 2}}{\rightleftharpoons} \alpha\text{-lactose monohydrate crystals}$$

The basis of this theory is that if the rate of crystallisation of α-lactose (step 2) is faster than the rate at which more α is available by mutarotation (step 1), then mutarotation is the rate

determining step for the overall crystallisation process. However, if step 2 is slower, then step 2 will be the rate determining step of the crystallisation process. In this case, the percentage of β- and α- anomers will be similar to their usual final equilibrium in water reported in literature i.e. 37% α and 63% β lactose (Twieg *et al.*, 1968).

The second hypothesis is based on the postulate that impurities can either inhibit or seed crystal growth depending on the materials involved (Ouiazzane *et al.*, 2008; Belhamri *et al.*, 2004). When crystallising α-lactose monohydrate, any traces of β-lactose present are considered as impurities. It was reported that the presence of β-lactose within a solution inhibits the crystal growth of α-lactose crystals (Eliasson, 2006; Hunzicker *et al.*, 1927).

The third hypothesis is based on the distinct variations in sweetness between both β- and α- anomers. As previously discussed (chapter 1), the enormous difference between the solubility of α-lactose monohydrate (7 g/100 mL) and β-lactose (50 g/100 mL) makes β-lactose much sweeter than the α-anomer. This, if not controlled, may lead to batch-to-batch irreproducibility of the chocolate crumb and even variations within the same batch.

The production of amorphous lactose will be accomplished by employing two methods: spray-drying and freeze-drying. Spray-drying has been reported to be capable of producing amorphous lactose successfully (Islam *et al.*, 2010; Langrish *et al.*, 2009; Whiteside *et al.*, 2008; Ibach *et al.*, 2007; Haque *et al.*, 2005; Buckton *et al.*, 2002). These studies proved the production of a high amorphous content of spray-dried lactose as confirmed by a variety of analytical techniques like PXRD, DSC, Fourier transfer infrared spectroscopy (FT-IR), and Fourier transfer Raman spectroscopy. However, the anomeric composition of β- and α- anomers of the spray-dried material was rarely reported. As the β/α anomeric ratio will impact on the crystallisation of lactose, it is important to establish a technique that can determine this ratio upon spray-drying.

Freeze-drying has also been successfully utilised for the production of amorphous lactose (Haque *et al.*, 2006; Thomsen *et al.*, 2005; Ottenhof *et al.*, 2003; Mazzobre *et al.*, 2001;

Roos, 1997). Analytical techniques like DSC, PXRD, FT-IR were employed to confirm a high amorphous content of the freeze-dried lactose material without a great concern about the β/α anomeric ratio.

In the project, freeze-drying will be used as the main technique for the production of amorphous lactose. This decision was considered for two reasons; one is that the yield produced by freeze-drying of lactose is greater than that obtained by spray-drying. Therefore, more amorphous lactose sample will be available for the analysis. The second is based on lines of investigation reported in literature which proved that spray-drying of lactose solutions (˃ 50 °C) influences the mutarotation and consequently changes the β/α ratio (Haque *et al.*, 2005; Hartel *et al.*, 1991), thus, causing saturation of lactose and the formation of small amounts of crystals in the amorphous sample which is not observed by freeze-drying.

The aim of this chapter is to establish a robust method for the production of amorphous lactose standards and to determine the anomeric purity of these standards, prior to readying them for incorporation into the model system (chapter 5).

To achieve the target of this chapter, the following objectives have been set:

1. Production of amorphous lactose. Two methods will be employed to fulfil the set target: spray-drying and freeze-drying.
2. Characterisation of stored amorphous lactose.
3. Determination of the β/α anomeric composition of lactose by NMR.
4. Determination of the β/α anomeric composition of lactose by optical rotation.

2.2 Materials and methods

The materials and equipment used in this chapter are: α-lactose monohydrate batch # L-3625 (Sigma Aldrich, UK); sucrose lot # 9069D5X (Silverspoon sugar); β-lactose Acros Organics batch # 41298-5000 (Fisher Scientific); phosphorous pentoxide P0679, purity ≥ 97% (Sigma-Aldrich, Gillingham, UK); sucrose ≥ 99.5 purity, batch # 077k00851 (Sigma Ultra);

indium purity 99.9999%, batch # 0160615 (Alfa Aesar, Heysham, UK); D-DMSO (dimethyl sulfoxide-D6) – D,99.9%, CAS # 2206-27-1 (Goss Scientific Instruments Ltd, UK); HPLC water batch # 0891818 (Fisher Scientific. UK); 5-place balance (Sartorius) serial # 20168766; magnetic stirrer serial # 14584377; Gilson pipette pipetman P5000 1-5 mls, F123603 (Anachem Ltd); Aldrich atmosbag zipper-lock, two hand, size L (Z530220-1EA) and size M (Z530204-1EA); BUCHI mini spray-dryer B-191 (Buchi Labortechnnik AG, Postfach, Switzerland); DMA 8000, serial # 533N8031701 (PerkinElmer, UK); VARIAN freeze-dryer serial # 19991339; NMR Bruker AV400; TGA 2950 equiped with gas cooling accessory (TA instruments, UK); DSC 2090 equipped with a refrigerated cooling system (TA instruments, UK); DSC Aluminium hermetic pans (batch # T081223) and hermetic lids (batch # T080730) (TA Instruments, UK); whirl mixer serial # 00051442 (Fisons); universal analysis software 2000 (TA Instruments, UK); topspin NMR software (Bruker, UK).

2.2.1 Preparation of the feed solutions for spray- and freeze-drying

For both spray- and freezing-drying, a 10% w/v aqueous feed solution of α-lactose monohydrate (where the actual weight of the α-anomer is 9.6 g corresponding to 96% α-lactose content as stated in the certificate of analysis) using crystalline material was prepared in HPLC grade water, this is a typical concentration used for these drying procedures (Ramos *et al.*, 2005; Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997).

All solutions were dissolved in glass beakers (200 mL for freeze- drying and 1 L for spray-drying) using a magnetic rotating stirring bar ~ 5 cm long. All solutions were prepared and stored at 25 °C, but two different protocols were employed. The first set of amorphous samples was prepared by starting the drying processes soon after dissolution of lactose (i.e. when no particulate matter was detected). For the 10% w/v solutions, the absence of particles was noted at 20 min after water was initially added to the lactose powder and so the drying commenced 30

min after initial mixing. A second protocol involved leaving the solutions 4 h after initial mixing before drying was initiated.

2.2.2 Spray-drying

Aqueous 10% w/v solutions of α-lactose monohydrate, (either the 30 min or 4 h solutions), were spray-dried using a Niro A/S micro-sprayer. The inlet temperature applied was 195 °C producing an outlet temperature of 95 ºC with a feeding rate of 20 mL/min and an air flow of 67 kg/h (Chidavaenzi *et al.*, 1997). This is a standard spray-drying preparation procedure for amorphous lactose given in the literature and is claimed to produce 100% amorphous material (Ramos *et al.*, 2005; Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997). The spray-drying process lasted a maximum of 50 min using 1 L of feed solution. The resulting spray-dried powders were collected from the cyclone sample chamber and placed immediately in a desiccator over P_2O_5, which was placed in a controlled-temperature room at 25 ºC for 48 h. This step allowed further drying of the amorphous material. After 48 h, the desiccator was placed in a disposable plastic glove bag (Aldrich Atmosbag, tape-seal, two-hand, non-sterile, size M, part number Z112818-1EA) filled with a continuous flow of nitrogen gas (oxygen free). Whilst in the bag and under nitrogen the whole powder sample was mixed and then divided into approximately 50 mg aliquots within 7 mL glass sample jars. The sample jars were sealed within the bag and placed back in a desiccator over P_2O_5. This procedure ensured that all of the samples were stored under nitrogen. Once loaded and sealed, the desiccator was placed back into the controlled-temperature room at 25 ºC. A sample jar was removed from the desiccator for each subsequent experiment and not replaced, i.e., a fresh sample was used each time in order to minimise the exposure of the powder to atmospheric humidity.

2.2.3 Freeze-drying

Aqueous 10% w/v solutions of α-lactose monohydrate (either pre-stored for 30 min or 4 h) were freeze-dried using a varian freeze-dryer Girovac model GVD4. Samples of lactose solution (2 mL) were placed in 7 mL glass sample jars, these sample jars were placed centrally on the shelf within the freeze-dryer. The freeze-drying process involved a pre-freezing step at - 80 ºC, a primary drying step for 72 h employing a temperature of - 50 ºC and vacuum pressure of 0.06 mbar, then secondary drying over P_2O_5 for 48 h at 25 ºC. The sample manipulation i.e. aliquoting, sealing and storage was conducted in a similar way to the spray-dried samples.

2.2.4 Powder X-ray diffraction (PXRD)

PXRD analyses were performed on a PANalytical CubiX PRO Fast diffractometer PW3800/00 with a Cu LFF X-ray tube PW3373/00 (PANalytical, Almelo the Netherlands). The powders were spread on a zero background holder and placed on a spinner stage with a spinner revolution time of 1 s. Bragg-Brentano geometry was applied using monochromatic Cu K alpha radiation and soller slits (0.02 rad) for both the incident and diffracted beam paths. The instrument was operated at a voltage of 45 kV and a current of 40 mA over a scan range 2-70 ° 2Theta with a step size of 0.0201 °2Theta and a time per step of either 71.75 s (Slow scan) and 8.89 s (Fast scan).

2.2.5 Differential scanning calorimetry (DSC)

The TA Q20 DSC was calibrated while operating the instrument in the calibration mode and applying a heating rate of 10 °C/min and the default nitrogen purge gas parameters. The baseline calibration was measured and corrected for the heat-flow deviation from zero of the DSC cell by running an empty cell through the chosen operating temperature range. The cell constant and onset slope were calculated by heating up indium pin-holed pan against a reference pin-holed empty pan at 10 ºC/min. Thereafter, the calibration was analysed according to a standard operating procedure SOP 2002/004 Manual set by KCL labs. The literature values of

the melting point of indium and the standard heat are 156.6 ºC and 28.71 J/g respectively. The cell constant was then calculated as the ratio of the measured enthalpy of fusion of indium and the literature value (Zeng, 1997). Additional DSC runs were made on an instrument equipped with a robot (Mettler DSC 823e) attached to Haake EK90/MT intracooler where dry N_2 was used as a purging gas, and 40uL Mettler Toledo hermetic aluminium DSC pans and lids.

All in all, the calibration of DSC was performed by scanning a thermogram of a pure substance of known melting point. Indium and tin are the most common calibrants used for DSC. One of the limitations of DSC is the selection of the appropriate temperature scanning rate as selecting a too fast scan rate can cause a broadening of the thermogram.

The sample was prepared by weighing 3 - 5 mg of the produced sugar into a DSC pan, a hermetically sealed pan with a pin-holed lid (to allow evaporation of any moisture embedded in the sample) was placed on the top of the pan and crimped to ensure that the pan was completely sealed. The DSC experiments were employed by equilibrating the system at 25 ºC, setting an isothermal mode for 10 minutes at 30 ºC followed by a ramping rate of 10 °C/min from 25 ºC to 200 ºC. The resulting graph was analysed by the universal analysis software 2000 (TA Instruments).

2.2.6 Dynamic mechanical analysis (DMA)

The DMA instrument was calibrated for the force component by following the manufacturer's manual (PerkinElmer). This was performed by tightening the stationary and the drive shaft sides of the cantilevers (with no pocket inside) and measuring the applied force. The temperature component was calibrated by indium with a melting point of 156.6 ºC and comparing the obtained value to the literature value.

The DMA samples were prepared by weighing 20 - 50 mg and loading them into a clean metal pocket made of a sheet of stainless steel then the powder pocket was folded to form an angle of 60° between the inner face of the pocket. The powder was then crimped to ensure that

the powder was fully compressed with the folded pocket and that a thin sandwich of 0.4 mm was formed. The sample was then clamped into the DMA instrument as a rectangular cross section in a single cantilever bending mode. One end of the pocket was clamped to the stationary side and the other end was undergoing an oscillating bending force through the drive shaft. The DMA furnace, which encased the clamping area completely, was used to apply the temperature program. The temperature of the sample was recorded by a platinum resistor sensor that is located behind the middle of the pocket (Royall *et al.*, 2005).

The experimental parameters employed in this test were a dynamic displacement of 0.05 mm, a multi-frequency mode of 1, 10 and 30 Hz and heating rate of 2 °C/min from 25 °C to 200 °C.

2.2.7 Thermogravimetric analysis (TGA)

A PerkinElmer Pyris 6 thermogravimetric analyser was calibrated for temperature and weight according to the methods supplied in the manufacturer's manual. Samples of 5-10 mg of lactose were loaded into an open pan and heated at a heating rate of 10 ºC/min over a temperature range from 25 ºC to 150 ºC, with the sample mass monitored as a function of temperature and time.

2.2.8 Proton nuclear magnetic resonance (H-NMR)

NMR samples were prepared by dissolving 3-4 mg of lactose in 0.7 mL of DMSO - Dimethyl Sulfoxide- d6 99.9% at %D with 0.05% v/v Trimethylsilane TMS (Goss Scientific instruments Ltd). 1-dimensional 1H spectra were recorded on a Bruker Avance 400-MHz spectrometer equipped with a QNP probe. The temperature was maintained at 298 K. Scans (n = 16) were recorded using a standard zg 30 pulse sequence (a 30 degree proton pulse followed by acquisition); with a 1 s recycle delay. The 30 degree pulse length was 3.43 microseconds. The sweep width was set to 20.69 ppm and the acquisition time was 3.96 s. Spectra were processed and analysed using the Bruker Topspin software. The free induction decay was multiplied with

an exponential function corresponding to a line broadening of 0.3 Hz, and spectra were phase corrected manually prior to an automatic baseline correction. Peaks were integrated using the β-lactose peak as a reference by defining its peak area as 1. The ratios of the two areas were compared following the method described by Willart *et al.*(2004). The TMS reference was used for comparing the chemical shifts.

2.2.9 Optical rotation

PerkinElmer polarimeter 343 was utilised to determine the optical rotation of lactose using a sodium D-line monochromatic radiation (λ = 589 nm) and a 1 dm cell path length. The instrument was first calibrated against two standard blank solutions: water and DMSO. Lactose samples were prepared at 25 ºC by dissolving lactose powder in HPLC grade water and DMSO at the following concentrations: 10% w/v (corresponding to the concentration used to prepare freeze dried and spray dried lactose) and 4% w/v (corresponding to the concentration used in previous polarimetry studies on lactose, Drapier-Beche *et al.*, 1999). After the initial mixing of the solution, the optical rotation was measured immediately after a clear solution was noted. Thus, for the 10% w/v solution optical rotation was measured after 20 min and for the 4% w/v after 10 min from the initial mixing. Measurements continued over 10-min time intervals for 400 min. The produced curve was fitted to an exponential decay and extrapolated to correct for the optical rotation at the initial time point.

2.2.10 Presentation of experimental data

Experimental data are expressed as mean ± standard deviations (SD). OriginPro 8 software was used for data analysis and fitting.

2.3 Results

The spray- and freeze-drying methods used in this study have all been reported at length within the literature and these are the standard preparations for producing highly amorphous forms of lactose (Ramos *et al.*, 2005; Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997). Many techniques that are highly sensitive towards measuring the amounts of disorder within materials have been used to confirm that the drying protocols reported here produced amorphous content approaching 100% (Haque *et al.*, 2005; Shah *et al.*, 2006; Royall *et al.*, 2005; Saunders *et al.*, 2004).

2.3.1 Characterisation of spray- and freeze-dried lactose by PXRD

The PXRD response of crystalline lactose samples used in this study agrees with the results reported in literature for the highly crystalline material (Shah *et al.*, 2006). The data for both α- and β- anomers (Figure 2.2) exhibited strong regular diffraction signals, particularly at 10.5° and 12.6°, which are characteristic of crystalline β-lactose and α-lactose monohydrate, respectively (Buckton *et al.*, 2002) while those corresponding to spray- and freeze-dried lactose exhibited a randomly dispersed diffraction pattern which is similar to the pattern of amorphous lactose reported in literature (Haque *et al.*, 2005). On this basis, the spray- and freeze-dried lactose fractions were assumed to be amorphous as the ordered regular pattern of diffraction peaks produced by crystalline materials (Figure 2.2) were obviated by processing. The PXRD results for both processed forms appeared to be identical.

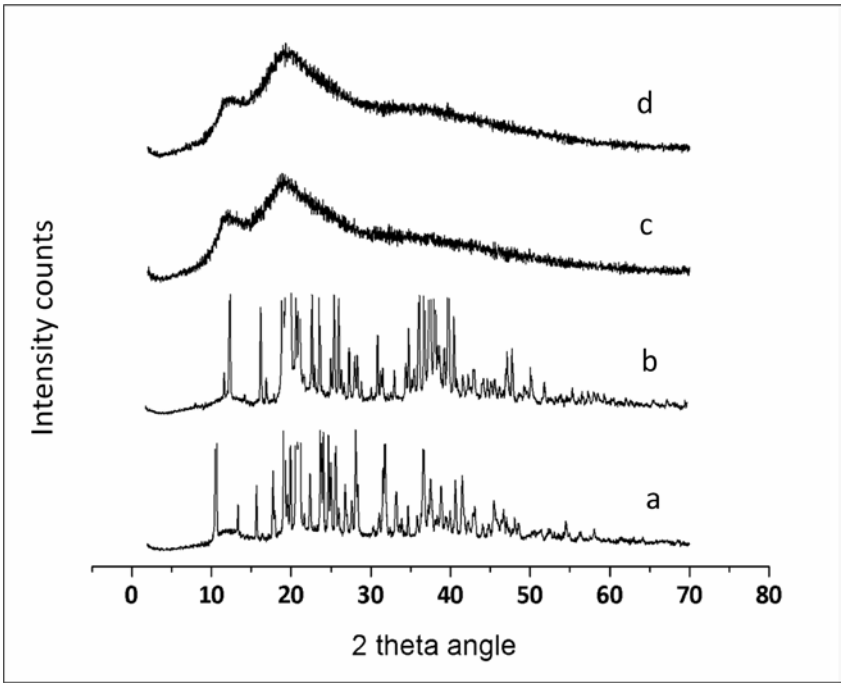

Figure 2.2 PXRD of lactose a) crystalline β-lactose (as received) b) crystalline α-lactose (as-received) c) freeze-dried lactose d) spray-dried lactose (both freeze-dried and spray-dried samples were analysed at t=0 time point where the feed solution was dried after 30 min.

2.3.2 Characterisation of spray- and freeze-dried lactose by DMA

The DMA thermograms of crystalline α-lactose monohydrate and the spray- or freeze-dried lactose samples were markedly different (Figure 2.3). The modulus signals for both the spray- and freeze-dried lactose samples were almost identical as a function of temperature. DMA is a technique that measures the modulus (strain/stress ratio) of a sample as an oscillating force is applied under the imposition of a controlled temperature programme. In principle, DMA obeys Hooke's law which states that a deformation or strain of a spring is linearly related to the force or stress applied by a constant specific to the spring.

The apparent modulus or hardness of the lactose samples were measured as the temperature was increased at a rate of 5°C/min (Figure 2.3). The crystalline sample showed little alteration in the modulus signal until the melting point, when the modulus dramatically collapsed. For the spray- and freeze-dried samples, a loss in the modulus of a similar magnitude

was observed just beyond 100 °C. This sudden decrease in the viscosity or hardness was associated with the glass transition of the dried materials, whereby at the glass transition the molecular mobility within the amorphous lactose powders increases rapidly (Royall *et al.*, 2005). The temperature at which the loss in modulus signal had reached its midpoint was determined to be approximately 120 °C, and this was designated as the glass transition temperature for the two forms of amorphous lactose used. An observed T_g of 120 °C falls within the glass transition range of amorphous lactose reported in literature (Hill *et al.*, 1998; Saunders *et al.*, 2004), which again implies that the spray- and freeze-dried samples were in the amorphous form.

On further heating, above the glass transition temperature, the amorphous lactose samples were in a liquid state with their molecules able to diffuse more rapidly and this increase in molecular mobility allowed crystallisation to begin (Hill *et al.*, 1998; Saunders *et al.*, 2004). Crystallisation was marked by the increase in modulus at approximately 150 °C. However, as the onset of melting approaches for these newly crystallised forms of lactose, the modulus falls again, producing a peak in the modulus profile. The large change in the value of the modulus at the glass transition (Figure 2.3) due to the high sensitivity of this method provides the key benefit of DMA when it is used to characterise amorphous powders (Royall *et al.*, 2005).

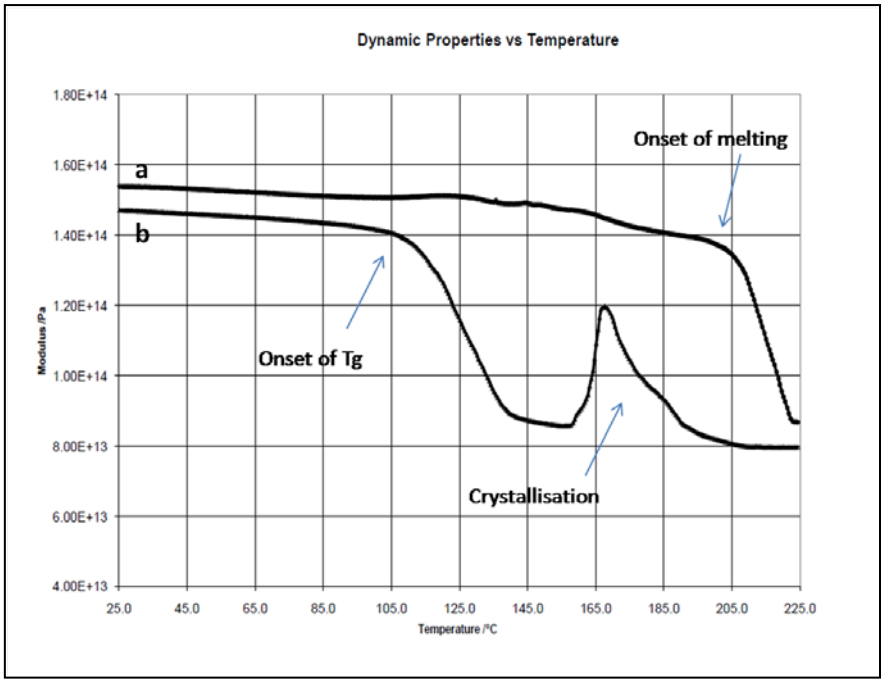

Figure 2.3 An overlay of DMA thermograms of lactose a) crystalline α-lactose monohydrate (as-received) and b) spray-dried lactose (dried after 30 min of the preparation of the feed solution).

2.3.3 Water content determination by TGA

The water content of both spray- and freeze-dried lactose was determined by TGA and was found to be 1.3 % ± 0.3 w/w and 1.4 % ± 0.3 w/w respectively (n= 3, ± SD). The % weight loss of water for both samples was determined by calculating the difference between the weight of the lactose sample at 25 °C and that at 120 °C where the baseline was stable, indicating no more water loss.

2.3.4 Characterisation of spray- and freeze-dried lactose by DSC

The DSC thermograms of the freeze-dried lactose revealed a decreasing step in the beginning of the thermogram which was due to the loss of the moisture embedded in the sample preceding freeze-drying. Upon heating, the freeze-dried amorphous lactose sample (with 4 h standing time of the feed solution prior to freeze-drying, and samples stored at a controlled temperature of 25 °C over P_2O_5) first showed a T_g baseline deflection which was clearly

observed as a step change between 110 - 120 ºC. The glass transition was analysed by plotting two tangent lines across the step change corresponding to the glass transition with the point of intersection of both tangents representing the glass transition temperature as recommended by Haines (2002) (Figure 2.4). The attained T_g agrees with the literature value which was documented to be 116 ºC (Roos, 1995). An exothermic peak was observed in the region between 174 -176 ºC which was designated as the re-crystallisation of amorphous lactose in the sample pan (Hill *et al.*, 1998) and subsequently a melting endotherm at ~ 206 ºC (Figure 2.5).

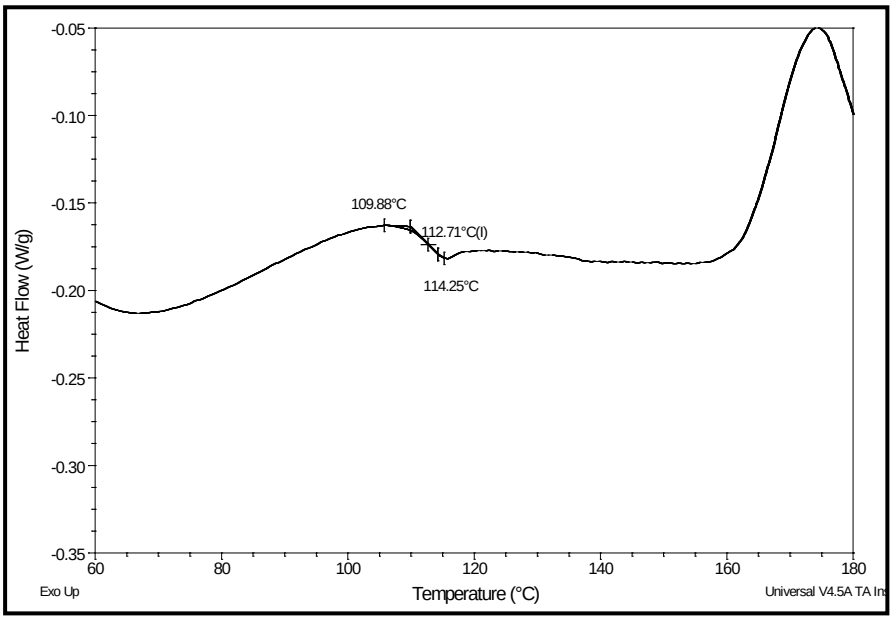

Figure 2.4 The glass transition analysis of freeze-dried lactose, by plotting two tangent lines across the step change corresponding to the glass transition with the point of intersection of both tangents representing the glass transition temperature as recommended by Haines (2002).

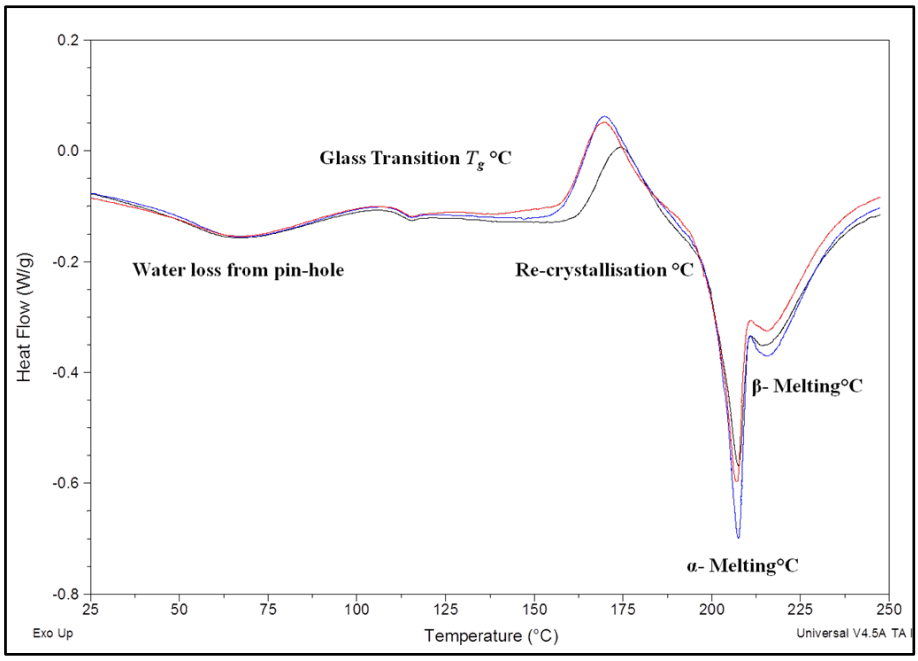

Figure 2.5 An overlay of DSC scans of freeze-dried lactose (three replicates) (analysed at t = 0 time point where the lactose solution was freeze-dried after 4 h of its preparation) where t=0 refers to analysis immediately after 48 h of secondary drying of the freeze-dried material over P_2O_5 at 25 °C.

Two consecutive melting peaks were detected in the DSC thermogram. It was reported that the first endothermic peak (210 - 220 °C) corresponds to the melting of the α-lactose monohydrate whereas the second endothermic peak (220 - 235 °C) corresponds to the melting of β-lactose (Islam *et al.*, 2010). α-Lactose was reported to clean melt at 202 - 210 °C while β-lactose melting point was 235 - 252 °C (Islam *et al.*, 2010; McSweeney *et al.*, 2009; Jorgensen *et al.*, 2006; Drapier-Beche, 1999; Simpson *et al.*, 1982; Lerk *et al.*, 1984; Figura *et al.*, 1995 and Garnier *et al.*, 2002).

Table 2.2 A summary of the glass transition, re-crystallisation and melting of freeze-dried lactose observed by DSC and analysed by Universal Analysis TA software (three replicates). 2-5 mg of sample were used per run; the freeze-dried lactose was analysed at t = 0 time point where the lactose solution was freeze-dried after 4 h of its preparation, where t=0 refers to analysis immediately after 48 h of secondary drying of the freeze-dried material over P_2O_5 at 25 °C.

		Re-crystallisation peak			**Melting peak**		
	T_g (ºC)	Onset Temperature °C	Peak Temperature ºC	Enthalpy (J/g)	Onset Temperature °C	Peak Temperature ºC	Enthalpy (J/g)
R 1	112.7	162.2	174.13	87.5	201.2	206.8	59.8
R 2	112.6	158.4	169.8	101	201.7	207.3	64.6
R 3	112.6	158.2	169.5	90.7	201.2	207.3	50
Mean	112.6	159.6	171.1	93.1	201.4	207.1	58.1
SD	0.1	2.3	2.6	7.1	0.3	0.3	7.4
%RSD	0.1	1.4	1.5	7.6	0.1	0.1	12.8

Three replicate samples were tested on DSC to check the reproducibility of the technique. The % RSD values tabulated in table 2.2 reflect a good repeatability which indicates that DSC can be a robust tool to detect the amorphicity of amorphous lactose.

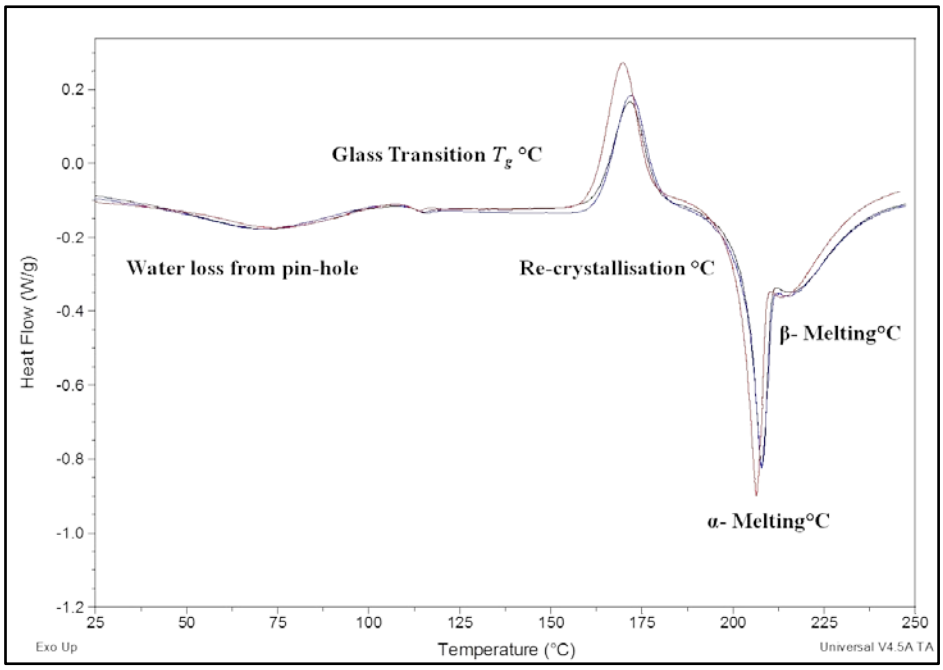

Figure 2.6 An overlay of DSC scans of spray-dried lactose (three replicates) (analysed at t = 0 time point where the lactose solution was spray-dried after 4 h of its preparation) where t=0 refers to analysis immediately after 48 h of drying of the spray-dried material over P_2O_5 at 25 °C.

Table 2.3 A summary of the glass transition, re-crystallisation and melting of spray-dried lactose observed by DSC and analysed by Universal Analysis TA software (three replicates). 2-5 mg of sample were used per run; the spray-dried lactose was analysed at t = 0 time point where the lactose solution was spray-dried after 4 h of its preparation, where t=0 refers to analysis immediately after 48 h of drying of the spray-dried material over P_2O_5 at 25 °C.

		Re-crystallisation Peak			**Melting Peak**		
	T_g (ºC)	Onset Temperature °C	Peak Temperature ºC	Enthalpy (J/g)	Onset Temperature °C	Peak Temperature ºC	Enthalpy (J/g)
R 1	112	161.3	169.5	118.9	202.3	206.3	85
R 2	113.6	163	171.9	109.7	203.9	207.7	75
R 3	112.9	162.5	171.9	99.8	203.6	2.07.8	72.9
Mean	112.8	162.3	171.1	109.5	203.3	207.0	77.6
SD	0.8	0.9	1.4	9.6	0.9	1.0	6.5
%RSD	0.7	0.5	0.8	8.7	0.4	0.5	8.3

2.3.5 Determination of the β/α anomeric ratio of spray- and freeze-dried lactose

The ^{1}H-NMR spectra of crystalline α-lactose, β-lactose, spray- and freeze-dried lactose were all similar apart from the region corresponding to approximately 6 ppm (Figures 2.7 & 2.8). ^{1}H-NMR analysis was performed to quantify the α- and β- anomers in lactose since the protons of the hydroxyl group (OH) at carbon C1 in the anomers are apparent at different chemical shifts (δ) due to their existence in different chemical environments. The characteristic ^{1}H-NMR signals corresponding to the α- and β- lactose anomers (Figures 2.7 & 2.8) appear as partially resolved doublets at 6.3 and 6.6 ppm respectively (Willart *et al.*, 2004).

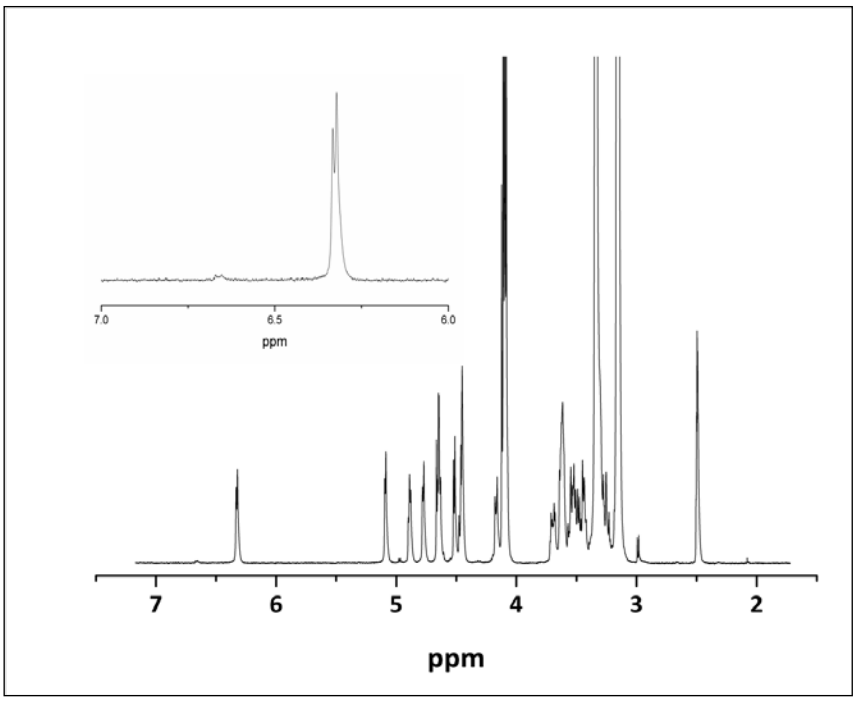

Figure 2.7 An NMR spectrum (400 MHz) of crystalline α-lactose solution as-received (0.7% w/v in DMSO). The inset shows the enlarged α- and β- anomer region (6-7 ppm).

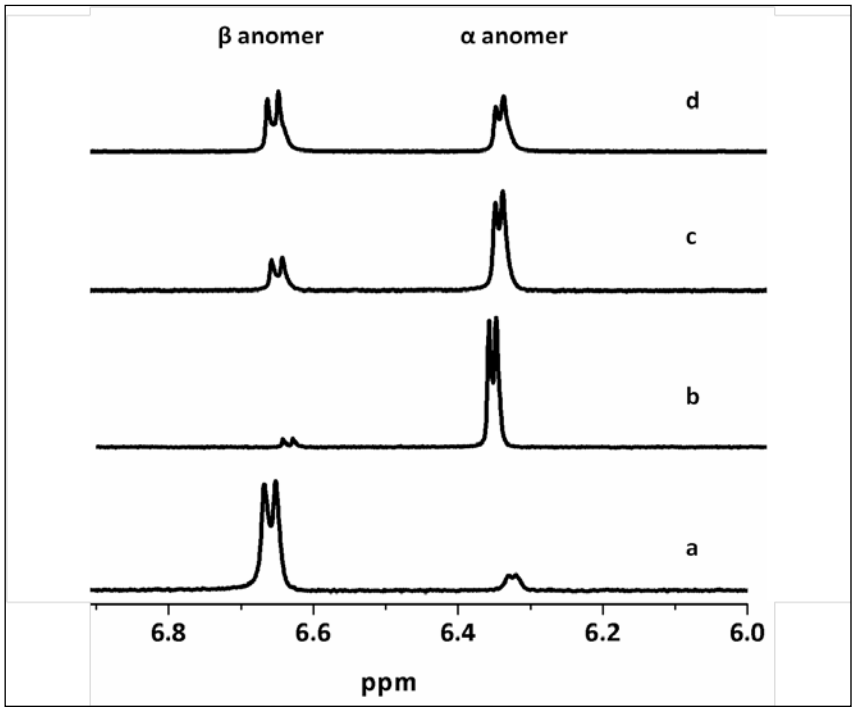

Figure 2.8 An overlay of NMR spectra of lactose a) crystalline β-lactose (as received) b) crystalline α-lactose monohydrate (as-received) c) freeze-dried lactose (analysed at t = 0 time point where the lactose solution was freeze-dried after 30 min of its preparation) d) spray-dried lactose (analysed at 0 time point where the lactose solution was spray-dried within 30 min of its preparation); t=0 refers to analysis immediately after 48 h of secondary drying of the spray- and freeze-dried material over P_2O_5.

The α- and β- protons (at C1) appear at the downfield of the ^{1}H-NMR spectrum as they are the most de-shielded atoms in the molecule. De-shielding takes place when the electron density, which shields a nucleus from the external field, on an atom is reduced by highly electronegative neighbouring groups. In the α- anomer, the distance between the proton at C1 and the neighbouring oxygen atoms is greater than that of the β- anomer (Figure 2.9). Thus, the influence of the neighbouring oxygen atoms on the protons at C1 of the β- anomer is greater causing a reduction of the electron density which leads to more de-shielding of the C1 proton of the β- anomer. This results in the chemical shift observed for β-anomer occurring at a higher value (δ= 6.6 ppm) than the chemical shift of the C1 proton associated with the α-anomer (δ=

6.3 ppm). The peaks appear as a doublet due to the spin-spin coupling resulting from the nearby hydrogen nuclei bound to C1 following the (n+1) rule. The peak areas of both signals were used to calculate the % of α- and β- lactose present. The ^{1}H-NMR spectra of both spray- and freeze-dried lactose showed that these different preparation methods produced lactose that exhibited different β/α ratios (Table 2.4, Figure 2.8). In addition, the ^{1}H-NMR analysis indicated that the crystalline α-lactose monohydrate was relatively pure with a 4% β and 96% α content while the β-lactose exhibited a composition of 87% β and 13% α.

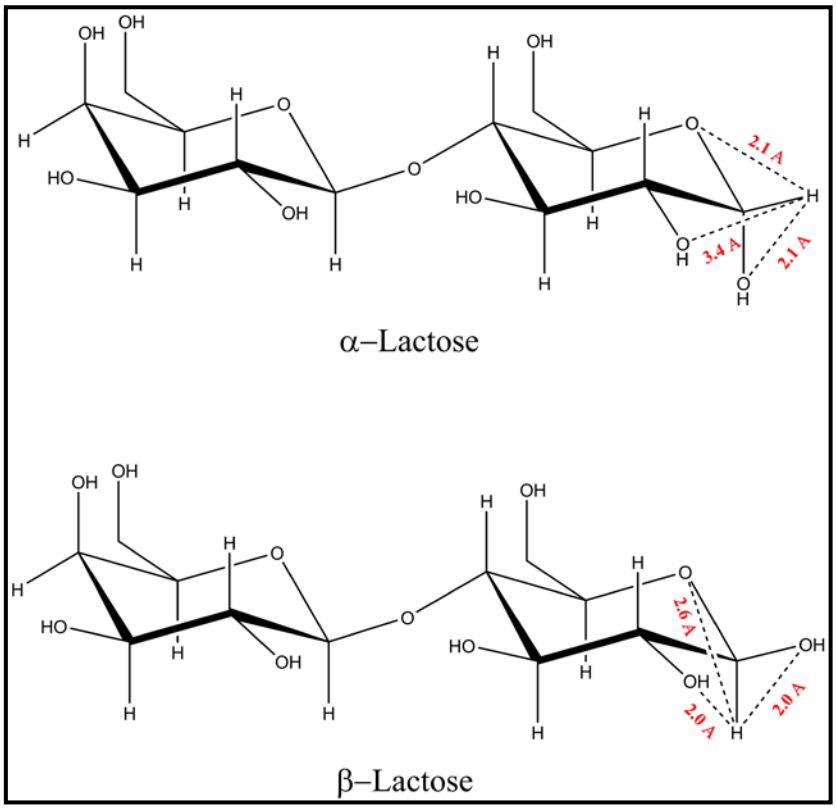

Figure 2.9 A Chemdraw® representation of the two anomers of lactose showing the difference in distance between C1 and its surrounding environment. Dimensions are based on the published crystal structure of α-lactose monohydrate (Clydesdale *et al.*, 1997).

The β/α ratio recorded for different standing or storage times of the feed solutions showed large differences. For example, freeze-drying within a 30 min standing time produced an anomer composition of 25% β and 75% α while a feed solution with a 4 h standing time produced a sample containing 60% β and 40% α. On the other hand, spray-drying within a 30

min standing time produced 52% β and 48% α while a feed solution with a 4 h standing time produced 57% β and 43% α. The standard deviation in the measured anomeric ratios for the spray- and freeze-dried lactose samples all fall within the 0.1 to 1.4 % range. The β/α ratio of freeze- and spray-dried lactose has also changed considerably upon storage, with the amount of the β- anomer increasing significantly during a 56 d storage period.

Table 2.4 The β/α ratio of different forms of lactose determined from the areas of the peaks attributed to the carbon 1 β- and α- protons. t=0 refers to analysis immediately after 48 h of secondary drying of the spray- and freeze-dried material over P_2O_5, t=56 d of secondary drying. (The SD of the β/α was calculated based on the individual β/α ratios calculated).

Lactose	% β ± SD	% α ± SD	β/α ± SD
Crystalline α	**4 ±1.4**	**96 ± 1.4**	**0.04 ±0.02**
Crystalline β	**87 ±1.2**	**13 ± 1.2**	**6.7 ± 0.02**
Freeze Dried (t=0) 30 min	**25 ± 1.0**	**75 ± 1.1**	**0.3 ± 0.02**
Freeze Dried (t=0) 4 h	**60 ± 0.1**	**40 ± 0.1**	**1.5 ± 0.01**
Freeze Dried (t=56 d) 30 min	**50 ± 0.5**	**50 ± 0.5**	**1.0 ± 0.02**
Spray Dried (t=0) 30 min	**52 ± 0.3**	**48 ± 0.3**	**1.1 ±0.01**
Spray dried (t=0) 4 h	**57 ± 0.1**	**43 ± 0.1**	**1.3 ±0.01**
Spray Dried (t=56 d) 30 min	**60 ±0.3**	**40 ± 0.3**	**1.5 ±0.01**

The mutarotation kinetics of the stored freeze-dried and spray-dried lactose were studied in the amorphous phase. The dried samples were tested by NMR at zero timepoint (which is directly after finishing the drying process) and at 56 d. The results are reported in table 2.4. The anomeric ratio was also measured at two further time points (in the middle of the 56 d frame)

which were 14 d and 28 d. The results showed a decreased pattern of the β/α ratio of freeze-dried lactose until the samples reached the equilibrium of β:α 50:50 at 56d.

In order to have more confidence in the reported data, it is believed that there should be more control of the experimental parameters such as temperature and humidity. Moreover, other analytical methods should have been employed to investigate the amorphicity of the stored dried samples; such techniques include DSC and/or PXRD. It is proven that the anomeric ratio changes in the solid-state as a function of time; yet the future work will be able to assess the kinetics or the pattern of this change.

2.3.6 Spray-drying optimisation

After the confirmation of the capability of spray-drying of the production of amorphous lactose, a method optimisation of lactose spray-drying has been performed. The main aim of this work was to investigate which parameter had the largest impact on the anomeric ratio, standing time, inlet/outlet temperatures or flow rate. Referring to the Niro manual, the main parameters that can be investigated were: inlet temperatures, outlet temperatures and flow rate. Therefore, these parameters were varied according to table 2.5.

Table 2.5 Method optimisation of spray-drying protocol, showing the different experimental conditions varied to optimise the β/α anomeric ratio of the spray-dried product.

Batch #	Time before feeding	Inlet temp °C	Outlet temp °C	Flow rate (mL/min)	Water content (% w/w) ± SD (n=2)	β/α	β/α ± SD (n=3)
A	30 min	195	95	20	1.3 ± 0.3	51.7/48.3	1.1 ± 0.01
B	4 h	195	95	20	1.3 ± 0.3	57.0/43.0	1.3 ± 0.01
C	4 h	190	90	20	2.1 ± 0.1	57.5/42.5	1.3 ± 0.01
D	4 h	200	100	20	1.3 ± 0.8	55.8/44.2	1.2 ± 0.01
E	4 h	210	80	40	1.4 ± 0.3	59.2/40.8	1.4 ± 0.01
F	4 h	195	100	10	1.6 ± 0.5	57.4/42.5	1.3 ± 0.01

The results show that there is no particular trend followed upon altering the inlet/outlet temperatures or the flow rate of the instrument. Thus, the parameters do not have a significant impact on the β/α ratio upon spray-drying using Niro. This can be a useful conclusion for pharmaceuticals. However, it favours using freeze-drying, in the project, for the production of amorphous lactose as the ratio of the β/α can be easily controlled.

2.3.7 Determination of the optical rotation by polarimetry

To validate the NMR experiments and confirm that negligible mutarotation took place whilst the samples were dissolved in DMSO, and a number of polarimetry experiments were conducted to determine the specific rotation of lactose solutions as a function of time. The observed specific rotation $[\alpha_{Obs}]_D$ normalises for solution concentration and pathlength. In this study it was calculated by applying equation 1 to the measured or observed optical rotation.

Equation 1

Where $[\alpha_{Obs}]_D$ = observed specific rotation, D = sodium D-line monochromatic radiation (λ = 589 nm), α = observed optical rotation, l = pathlength in dm, C = concentration in g/100 mL

The polarimetry and ^{1}H-NMR data (Table 2.6) showed that for up to 30 min after the DMSO solution had been prepared there was no change in either the anomeric ratio determined by ^{1}H-NMR or the optical rotation of the DMSO solution prepared from crystalline α-lactose monohydrate. In further polarimetry experiments, the optical rotation began to decrease at a low rate only after 40 min. All of the ^{1}H-NMR experiments were conducted within 20 min of the DMSO solution being prepared. Therefore it was assumed that the anomeric ratios determined by the ^{1}H-NMR experiments reported here are true reflections of the composition within the solid phase. Thus the assumption that DMSO has little effect on the mutarotation of lactose was proven.

Table 2.6 The observed specific rotation and anomer concentration determined by ^{1}H-NMR for 0.7% DMSO solutions of α-lactose monohydrate, (certificate of analysis 96% w/w α and 4% w/w β supplied by Sigma Aldrich). Values determined as a function of time from solution preparation.

Time (min) from solution preparation	Optical Rotation	Anomer content determined by ^{1}H-NMR	
	$[\alpha]_D$	% w/w β ± SD	% w/w α ± SD
0	88.3°	4.2 ± 1.4 (n=6)	95.8 ± 1.4 (n=6)
10	88.1°	-	-
20	87.8°	-	-
30	87.7°	3.9 ± 0.1 (n=2)	96.1 ± 0.1 (n=2)

The specific rotations of 4% and 10% w/v aqueous solutions prepared using crystalline α-lactose monohydrate were also determined by polarimetry to establish the time required for the lactose solutions to reach anomeric equilibrium. The optical rotation values of the lactose solutions were recorded over a period of 400 min (Figure 2.10) and the results showed that the solution took approximately 4 h (240 min) to reach its equilibrium composition of 63% β and 37% α (Fox *et al.,* 1998; Fox *et al.*, 2000). The equilibration time was taken to be the time required to reach a plateau or a constant value for the observed specific rotation.

The first step in the conversion of specific rotation data into the relative fractions of the two anomers was the extrapolation of the measured specific rotation to zero time after mixing (Figure 2.10) i.e. to the theoretical point where the optical rotation is measured on a freshly prepared solution. This extrapolation was carried out by fitting the experimental data to a first order exponential decay, with the fits for all data sets having $r^2 > 0.999$. The observed specific rotation of α-lactose monohydrate at this extrapolated intercept was determined for a 4% w/v solution to be 82.6°. This value is the specific optical rotation associated with the sample of α-lactose monohydrate before any mutarotation could have occurred. From the independent NMR results reported above, this sample was found to comprise 4% β and 96% α,

which precisely correlates with the stated purity provided by the suppliers. Thus, it could be concluded that a sample of lactose containing 96% of the α-anomer has an optical rotation of 82.6°. The other point where the anomer ratio is unambiguously known is at equilibrium in aqueous solution, the plateau region on figure 2.10. The anomer ratio at this point has been reported as 63% β and 37% α (Fox *et al.*, 1998; Fox *et al.*, 2000) and the specific rotation for this system was measured as 50.6°, the average specific rotation measured for the solution after 4 h.

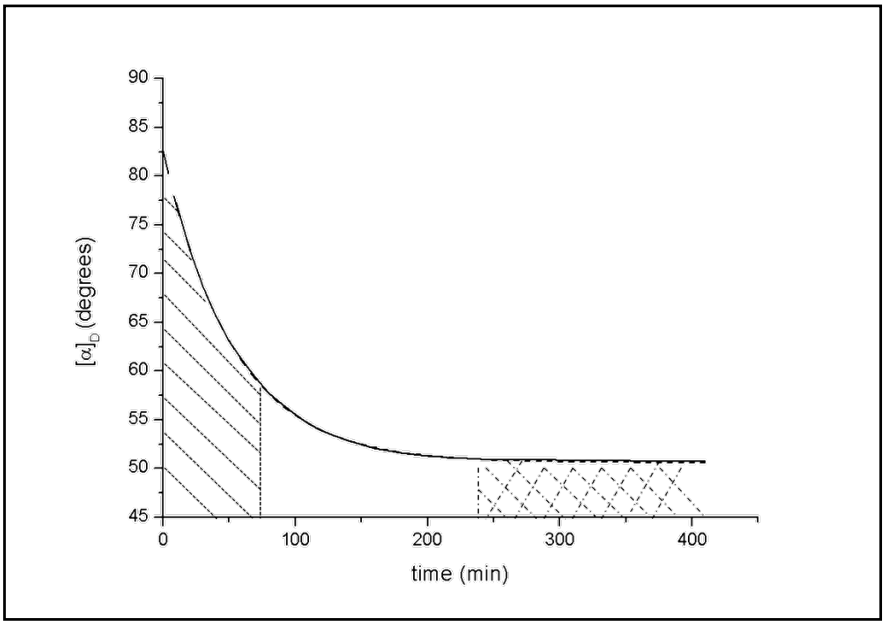

Figure 2.10 An optical rotation plot of 4 % w/v α-lactose monohydrate in water (fitted to exponential decay), experimental parameters are λ= 589 nm, 10 cm pathlength and 25 °C; single hatch - time frame when spray-drying took place; cross hatch - time frame when equilibrium ratio is attained.

To aid the determination of the anomeric ratio from optical rotation measurements, the following constants and variables were defined:

$[\alpha_{\alpha MH}]°$ was defined as the specific optical rotation of 100% pure α-lactose monohydrate that has suffered no mutarotation, likewise;

$[\alpha_{\beta}]°$ was defined as the specific optical rotation of 100% pure β-lactose that has suffered no mutarotation.

$[\alpha_{Obs}]_D$ was defined as the observed specific optical rotation for any sample of lactose under study. A measured experimental value therefore was a known dependent variable.

f_α was defined as the fraction of α-lactose present in the aqueous solution, a variable which depends on time in solution, temperature, concentration and pH. The value of this variable will lie between 0 and 1.

Similarly, f_β was defined as the fraction of β-lactose present in the aqueous solution.

These parameters are related by the following equation.

$$[\alpha_{Obs}]_D = (f_\alpha \times [\alpha_{\alpha MH}]^\circ) + (f_\beta \times [\alpha_\beta]^\circ) \qquad \text{Equation 3}$$

The aim of the optical rotation work was to determine the amount of β- and α-lactose present in aqueous solution as a function of time. Therefore, for the two special cases discussed above, for the pure α-lactose monohydrate at time 0 (extrapolated) and after 4 h equilibration, the known values for the composition and the measured optical rotation were substituted into equation 2 to produce the following simultaneous equations. It should be noted that a sample containing 96% α-lactose monohydrate and 4% β-lactose, will have f_α and f_β values of 0.96 and 0.04 respectively.

At time 0 for a 4% w/v solution;

$$82.6 = (0.96 \times [\alpha_{\alpha MH}]^\circ) + (0.04 \times [\alpha_\beta]^\circ) \qquad \text{Equation 4}$$

At equilibrium after 4 h for a 4% w/v solution;

$$50.6 = (0.37 \times [\alpha_{\alpha MH}]^\circ) + (0.63 \times [\alpha_\beta]^\circ) \qquad \text{Equation 5}$$

For these experimental conditions $[\alpha_{\alpha MH}]^\circ$ and $[\alpha_\beta]^\circ$ are constant, so the two simultaneous equations 4 & 5 may be solved to determine the values for $[\alpha_{\alpha MH}]^\circ$ and $[\alpha_\beta]^\circ$. This gave a value for $[\alpha_{\alpha MH}]^\circ$ of 84.8° and $[\alpha_\beta]^\circ$ of 30.5°. These values are close to the literature values reported for 4% w/v aqueous solutions of the pure forms of α-lactose monohydrate and β-lactose, which are 84° and 34° respectively (Drapier-Beche *et al.*, 1999). This calculation described above was also repeated for the 10% w/v solution, the results are listed in table 2.7 showing that as the concentration is decreased, the optical rotation for the pure forms of lactose increases.

Table 2.7 The extrapolated time zero (t =0) observed specific rotation $[\alpha_{Obs}]_D$ and the specific optical rotation for pure α- and β-lactose determined for two different concentrations of α-lactose monohydrate aqueous solutions.

Concentration % (g/100 mL)	$[\alpha_{Obs}]_D$ at t=0	$[\alpha_{\alpha MH}]°$	$[\alpha_\beta]°$
4	82.6°	84.8°	30.5°
10	78.0°	80.0°	30.7°

Once $[\alpha_{\alpha MH}]°$ and $[\alpha_\beta]°$ were determined experimentally, equation 3 was re-arranged to solve for both f_α and f_β. This re-arrangement exploits the fact that $f_\alpha + f_\beta = 1$.

$$f_\alpha = ([\alpha_\beta]° - [\alpha_{Obs}]_D)/([\alpha_\beta]° - [\alpha_{\alpha MH}]°) \quad \text{Equation 6}$$

$$f_\beta = ([\alpha_{\alpha MH}]° - [\alpha_{Obs}]_D)/([\alpha_{\alpha MH}]° - [\alpha_\beta]°) \quad \text{Equation 7}$$

Equations 6 and 7 may be applied to lactose polarimetry data in order to determine the fractions of the anomers present in solution at any time point by simply measuring $[\alpha_{Obs}]_D$ at the time of interest. However, it should be noted that equations 6 and 7 are only applicable if the experiments are conducted under the same conditions and concentrations as used for the determination of $[\alpha_{\alpha MH}]°$ and $[\alpha_\beta]°$ values. In the present study, equations 6 and 7 were used to determine the composition of the 10% w/v feed solutions used for the spray- and freeze-drying at important time points. For example, for a 10% w/v aqueous solution of Sigma-Aldrich supplied α-lactose monohydrate, containing 4% β and 96% α, 30 min after preparation, the solution contains 30% β and 70% α, and after 4 h it contains 63% β and 37% α which is a β/α ratio of 1.7. The half-life to reach the equilibrium composition for a 10% w/v solution was 36 min and it took 4 h to reach the steady-state equilibrium composition of anomers. Therefore in order to control the anomer composition present in spray- and freeze-dried lactose it would be prudent to monitor and control the standing time of the feed lactose solutions.

2.4 Discussion

The aims of this chapter were to establish a reproducible protocol for producing amorphous lactose and determine its anomeric purity. To achieve these aims, three main objectives were set. The first objective was to produce amorphous lactose either by spray- or freeze-drying. The second was to determine the anomeric purity of crystalline lactose used as a starting material for the production of amorphous lactose. The third objective was to determine the purity of the produced amorphous lactose.

The amorphicity of the spray- and freeze-dried samples of lactose was confirmed by DSC, DMA and PXRD. The DSC thermograms showed a glass transition at ~ 112 °C which agrees with the reference values reported in literature. As the sample was heated up, the mobility of the molecules gradually increased thus initiating nucleation and crystal growth. Ultimately, re-crystallisation occurs followed by melting of the sample. Therefore, DSC was able to characterise the freeze-dried lactose with high amorphous content. An important observation in the DSC results of both spray- and freeze-dried lactose is that the enthalpy of re-crystallisation as well as the heat of melting of freeze- and spray-dried lactose show slight differences. For example, the enthalpy of re-crystallisation of freeze-dried lactose is 93.1 J/g ± 7.1 (± SD, n=3) while the enthalpy of re-crystallisation of spray-dried lactose is 109.5 ± 9.6 (± SD, n = 3) (Tables 2.2 & 2.3). This might be due to the fact that spray- and freeze-dried lactose are able to re-crystallise into different crystal forms, which in turn, have different enthalpies of re-crystallisation (Haque *et al.*, 2005). It was also proven that the type of crystals formed highly depend on the drying method used (Drapier-Beche *et al.*, 1997; Jouppila *et al.*, 1997; Wursch *et al.*, 1984). For example, spray-dried lactose may crystallise to a mixture of α-lactose monohydrate and β-lactose (Bushill *et al.*, 1965; Briggner *et al.*, 1994) or only β-lactose crystalline (Wursch *et al.*, 1984) or α-lactose monohydrate crystalline (Aguilar *et al.*, 1994); whereas, freeze-dried lactose may re-crystallise into α-lactose monohydrate or β-lactose (Jouppila *et al.*, 1998; Drapier-Beche *et al.*, 1997; Bushill *et al.*, 1965) or to an anhydrous mixture of α- and β-lactose (Jouppila *et al.*, 1998).

The DMA thermograms showed a glass transition at approximately 120 ºC with a large decrease in the storage modulus when samples of both spray- and freeze-dried lactose were heated. The decrease in the resistance of the sample to deformation was attributable to the de-vitrification of the processed samples (Jones, 1999) but the storage modulus in the test samples was not completely lost because of the underlying contribution from the stainless steel of the powder pocket (Royall *et al.*, 2005). However, immediately after the glass transition, the modulus was reduced to a similar value to that observed after the melt associated with the crystalline material. The similar modulus values post melt and glass transition indicates that all of the spray- and freeze-dried material was amorphous. If any crystalline material had been present in the processed material, this would have made a contribution to the signal, giving an intermediate value in the modulus until the melting point of any residual crystalline material had been reached.

It was observed that the T_g produced by DMA (T_g = 120 °C) was slightly different from that produced by DSC (T_g = 112 °C). This observation was explained by Royall *et al.* (2005) who attributed the difference in the T_g values of amorphous lactose samples when run on DSC and DMA, to the difference in the water loss of the sample. As the plasticised amorphous lactose (containing more water) will have a lower T_g value. Therefore, the sample that will lose water slower will be plasticised and consequently will exhibit a lower T_g , in this case DSC.

The PXRD data presented within this chapter not only clearly shows the key benefits of this important technique but also highlights its central limitation. PXRD is reported to be the gold standard for amorphous detection (Shah *et al.*, 2006; Saunders *et al.*, 2004; Lehto *et al.*, 2006). However, PXRD is unable to characterise mutarotation within amorphous materials and therefore additional techniques require development.

The ^{1}H-NMR using a DMSO solution based sample preparation method developed here is a simple and sensitive method for characterising the anomeric composition within lactose samples that are either amorphous or crystalline. Polarimetry has supported the hypothesis that DMSO as an aprotic solvent has little impact on mutarotation. When a lactose solution in

DMSO is analysed within 20 min of preparation, the NMR spectra has been shown to reflect the anomeric composition present in the solid material (Table 2.6). Therefore, a key aim of this study has been achieved by establishing a quantitative measurement by NMR of the α- and β- anomer content in amorphous lactose.

The ^{1}H-NMR method identified an identical anomer composition of the Sigma supplied crystalline α-lactose monohydrate as that stated on its certificate of analysis. A GC method was reported in the certificate of analysis, thus the NMR method described here has been validated employing a second method. In addition, the composition of the supplied crystalline α-lactose monohydrate determined by polarimetry for a freshly prepared solution also matched the certification of analysis and NMR determined compositions.

The standard deviation of the α- content for the supplied crystalline α-lactose monohydrate sample, determined by NMR was 1.4% w/w (n = 6), which was larger than the value of 0.1% w/w (n = 6), determined for both the spray- and freeze-dried samples produced from fully-equilibrated feed solutions (t = 4 h). For the crystalline and amorphous samples, the NMR analysis was conducted on a number of different days, to investigate and accommodate the impact of any potential instrumental drift on the variability in response. The spray- and freeze-dried materials were both prepared in 2 batches and 3 samples from each batch were analysed, so as to comprise the 6 replicates for each type of dried material (Table 2.4). These samples were handled with great care, ensuring thorough mixing and storage under a nitrogen atmosphere at 0% RH and 25°C. Thus, the narrow standard deviation was brought about by the tight control of the analyte preparative conditions. Since crystalline α-lactose monohydrate is relatively stable, it was considered unnecessary to store these samples under such tight control. Six samples were analysed from the Sigma supplied batch stored under ambient conditions. However, it appears that fluctuations in the homogeneity and storage conditions of the crystalline α-lactose monohydrate did influence the standard deviation of the determined anomer composition, while its overall composition also measured by NMR matched the supplied

certificate of analysis. The standard deviations for the measured compositions reported here are much lower than the error range obtained from the solid state ^{13}C-NMR approach (of almost 10%) described by Lefort *et al.*, 2006. These data provide further justification for the development and use of the solution based ^{1}H-NMR analytical approach.

Combining the NMR data with the results from the polarimetry may be used to reveal the origins of the varying anomeric composition of amorphous lactose. The standing time for the feed solutions used for both freeze- and spray-dried lactose was found to have a large impact on the anomer composition of the final amorphous product. For both a 4% and a 10% w/v aqueous solution prepared from a typical batch of crystalline α-lactose monohydrate, there is a rapid change in the anomer content over the first 4 h. After this time, mutarotation reaches equilibrium and a steady-state or constant anomer composition was attained (Figure 2.10). Thus, spray- or freezing-drying a solution of lactose 4 h after preparation of the solution led to nearly a 35% w/w difference in anomer composition within the solid amorphous material compared to samples dried within 30 min of preparation. A contributing factor to this difference in anomer composition is clearly the mutarotation taking place in the lactose feed solutions, an observation that has received little attention in pharmaceutical literature.

The two concentrations of lactose in solution, 4% and 10% w/v, used in the polarimetry experiments were selected to mirror the concentrations used in previous polarimetry experiments (Drapier-Beche *et al.*, 1999) and to represent typical values used in spray- and freeze-drying (Haque *et al.*, 2005; Shah *et al.*, 2006; Royall *et al.*, 2005; Saunders *et al.*, 2004). For the 4% w/v solution, the $[\alpha_{\alpha MH}]°$ and $[\alpha_{\beta}]°$ values were very close to those reported by Drapier-Beche *et al.*, 1999 (Tables 2.1 & 2.6). Incorporating the $[\alpha_{\alpha MH}]°$ and $[\alpha_{\beta}]°$ values calculated for the 10% w/v solution into equations 6 and 7 allowed the determination of the anomer composition for the freshly-prepared solution, 30 min later, and 4 h after the formation of the solution from the observed specific rotations measured in the polarimeter. The anomer content of the freeze-dried lactose reflects most closely the extent of mutarotation observed in solution. A 10% w/v α-

lactose monohydrate aqueous solution at 30 min after initial mixing contains, from the polarimetry reported here, 30% β and 70% α, and the equivalent freeze-dried sample prepared at 30 min contained 25% β and 75% α as measured by NMR (Table 2.4).

Freeze-dried amorphous lactose dried within 30 min of solution preparation contains a high α-lactose content, whereas a solution held at 25 °C for 4 h and then freeze-dried, the resulting solid has a high β-lactose fraction (Table 2.4). The actual amounts are within 3-5% of the anomer composition observed in solution at these time intervals by polarimetry. The initial stage of freeze-drying, pre-freezing at - 80 ºC is very rapid and therefore this process must capture and preserve the degree of mutarotation within the solution, with minimal mutarotation possible in the glassy matrix once the ice crystals have formed. The rate of mutarotation is known to slow dramatically as the temperature is lowered (Haase *et al.*, 1966; Roetman *et al.*, 1975). The following stage of the process, the low primary drying temperature of - 50 ºC also negates mutarotation within the lactose-water glassy matrix. Therefore, the β/α anomeric composition of the initial feed solution was found to be very similar to the anomeric composition of the final amorphous cake for freeze-dried lactose.

When the feed solution for the spray-dryer is held for 4 h at 25 ºC before processing, the resulting material broadly conforms to the findings obtained for the freeze-dried samples, i.e. the solution β/α anomeric ratio reflects the composition of the resulting solid amorphous material. However, when the feed solution is spray-dried within 30 min of preparation, the amount of the β- anomer is much greater than predicted by considering the mutarotation occurring in solution as a function of time alone. Haase *et al.* (1966) recorded an increase in the rate constants for lactose mutarotation as a function of temperature. Thus, the 195 ºC inlet temperature of the spray-drier, even though the lactose solution is only exposed to this temperature for a few seconds, would be expected to accelerate the process of mutarotation compared to the feed storage temperature of 25 ºC. This would result in the presence of a higher amount of the β-anomer. The influence of the inlet temperature on solutions that have been equilibrated for 4 h

at 25 ºC is less as these systems have reached their anomeric equilibrium, i.e. they already have a large proportion of β- lactose present.

The time taken for spray-drying to be carried out would also be expected to have a major impact on the β/α anomeric composition of the spray-dried material prepared from the feed solutions dried within 30 min of solution preparation. The time taken to spray-dry one litre of feed solution was approximately 50 min. Since spray-drying was begun within the 30 min window, the last portion of feed solution was not processed until 80 min after the preparation of solution. This 80 minute zone (Figure 2.10) is the most dynamic part of the mutarotation equilibrium process such that at the end of this period the amount of β-lactose present in the feed solution would be high and nearing its 63% concentration at equilibrium. Therefore, when all of the spray-dried lactose from the 30 min feed solution is collected and mixed; the β-lactose content is higher, 57%, compared to the 30% β-lactose content of the aqueous solutions measured at 30 min by polarimetry.

It is worth noting here that the method optimisation of the Niro spray-drying of lactose has proved that altering the parameters of the Niro spray-dryer e.g. inlet/outlet temperatures or the flow rate of the instrument did not have any significant impact on the final β/α ratio of the spray-dried lactose. This might be due to the exposure of the lactose feed solution to high temperatures for almost 1 h (the time needed to Niro spray-dry 1 L of feed solution), which leads to that all the feed solutions are heading towards a similar final equilibirium where the only difference noticed was when altering the standing time and this can be clearly explained by figure 2.10.

The solubility of α-lactose monohydrate has been rarely addressed in the literature associated with spray- and freeze-drying (Chidavaenzi *et al.*, 1997; Listiohadi *et al.*, 2009). The solubility of α-lactose monohydrate is 7 g/100 mL (Fox *et al.*, 1998); in many optical rotation studies, which typically use a 4% w/v solution, the solubility is not exceeded (Drapier-Beche *et al.*, 1999). In contrast, the 10% w/v solutions typically used for spray- and freeze-drying do

exceed the solubility of α-lactose monohydrate in water (Fox *et al.*, 1998; Chidavaenzi *et al.*, 1997; Listiohadi *et al.*, 2009). However, mutarotation enhances the observed solubility because as the α-lactose monohydrate dissolves into water, then the β-anomer is rapidly formed, causing a reduction in the α- anomer's concentration in water and therefore increasing the observed solubility until a final equilibrium is eventually reached in solution. The consequence of the initial rapid conversion to the β-anomer is that the half-lives taken to reach anomeric equilibrium are similar for solutions initially prepared from 10 g and 4 g lactose per 100 mL of water (36 min). Therefore, using feed solutions which just exceed the solubility limit of 7% w/v is expected to have minimal impact on the observed β/α anomeric ratio of the resulting amorphous lactose produced by both spray- and freeze-drying.

Mutarotation was also found to occur in the solid amorphous lactose over a 56 d storage period (Table 2.4), for example the 30 min freeze-dried samples when stored for this period suffered a 25% increase in their β-anomer content. Lefort *et al.*(2006) also reported an increase in the β-anomer via solid-state mutarotation, which was initiated by heating amorphous lactose to temperatures close its glass transition, whereas no significant mutarotation was detected at 25°C. Their postulate was that the mechanism of solid-state mutarotation is different from that observed in aqueous solution as water was excluded from their milled material. However, in the current work 1.3 and 1.4% w/w water was found to be present within the stored amorphous lactose. For both studies, no re-crystallisation was detected over the time frame of the experiments, which for the Lefort *et al.* study was up to 4 h and for the current study reported here was 56 d. It would have been interesting if in the earlier study (Lefort *et al.*, 2006) the experiments had been repeated after an extended period of isothermal storage.

In aqueous solution, mutarotation follows apparent first order kinetics for reversible reactions approaching equilibrium (Haase *et al.*, 1966) observed here by the consistency of the half-lives measured at different concentrations. At 25 °C, as a consequence of mutarotation, the anomeric composition reaches a constant β/α ratio of 1.7 after 4 h. In the amorphous state and

in the presence of small amounts of water (1.3 - 1.4% w/w) the mutarotation of lactose continues towards this 1.7 ratio (Table 2.4), however it takes much longer to achieve. For example, after 56 d storage at 25 °C the composition of the 30 min spray-dried lactose samples had moved close to the 1.7 ratio with a content of 60% β and 40% α (Table 2.4). The presence of the small amount of water within the amorphous lactose samples prepared in this work indicates that the mutarotation mechanism shown in figure 2.1 may still be applicable when considering the spray- and freeze-dried materials. This finding does not contradict the earlier work (lefort *et al.*, 2006) as it was not clear whether the anomeric equilibrium had been achieved in these studies.

The dynamic nature of the β/α composition for amorphous lactose produced by different methods and stored for different lengths of time has a significant impact on the applications of lactose in pharmaceutical science. The two anomers possess a range of physicochemical properties (McSweeney *et al.*, 2009; Drapier-Beche *et al.*, 1999) and thus recording and maintaining the anomeric composition will be essential for the control of the model system that will be discussed in chapter 5, and may contain up to 34% w/w lactose.

2.5 Conclusion

This study has shown that the β/α ratio for amorphous lactose is not constant. It depends on both the production method and storage conditions. These findings impact on the use of amorphous lactose in medicines and as a calibrant for amorphous content determination**.** The aim of the work was accomplished; a simple solution based ^{1}H-NMR method has been established which can measure the β/α anomer composition of spray- and freeze-dried amorphous lactose with a standard deviation as low as 0.1% w/w (n = 6).

The ^{1}H-NMR method described here has given important insights into the properties and production of amorphous lactose. The control of the storage conditions of amorphous lactose is vital, as the α- to β- mutarotation continues even in the solid amorphous form. A

contributing factor to the wide ranging anomeric composition of amorphous lactose reported in literature (Ramos *et al.*, 2005; Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997; Buckton *et al.*, 2002; Listiohadi *et al.*, 2009) is a poor appreciation of the mutarotation equilibrium within the feed solutions prior to initiation of the drying processes. When lactose is in aqueous solution, its β/α content approaches a ratio of 1.7. Therefore it is recommended that in order to produce a consistent anomer composition within spray- and freeze-dried amorphous lactose, the standing time for the feed solution should be greater than 4 h, so that the solution is well-removed from the most dynamic region of the mutarotation profile, (single hatched area on figure 2.9) and within the plateau region (double hatched area on figure 2.9) so that the equilibrium content of 63% β and 37% α is approached. If the amorphous material has been formed from a solution that has not been allowed to stand for 4 h, the resulting solid will continue to undergo slow mutarotation should trace amounts of moisture be present, with the anomeric β/α ratio also tending towards 1.7 over a number of weeks of storage.

Chapter three – Epimerisation of α-lactose monohydrate in aqueous solution

The aim of this chapter is to investigate the epimerisation kinetics of α-lactose monohydrate at different temperatures. This will involve the determination of the overall rate constant of the reversible epimerisation reaction, the forward and reverse rate constants.

3.1 Introduction

Lactose can exist in two isomeric forms called anomers. These anomers are either α- or β- anomer. They differ by the conformation of the hydroxyl group at the anomeric Carbon C1 of the glucopyranose ring (McSweeney *et al.*, 2009). The phenomenon of transformation from one anomer into its chiral counterpart is called epimerisation (McSweeney *et al.*, 2009). This process of interconversion between both anomers is commonly referred to as mutarotation. This is considered as a misuse of the term mutarotation in modern litertuare. Mutarotation refers to a time dependant rotation of the plane of linearly polarised light (McSweeney *et al.*, 2009). This can not be measured with practical reliability for solid samples. Therefore, it is more appropriate and meaningful to refer to the phrase 'epimerisation' rather than 'mutarotation'. Thus, the term epimerisation will be used in this chapter to express the interconversion from α-lactose to β-lactose.

Epimerisation can lead to the existence of different forms of crystalline states of lactose including α-lactose monohydrate, α-lactose anhydrous, β-lactose, and different co-crystal systems with α- and β- in different stoichiometric ratios (Jawad *et al.*, 2012; Jarring, Astrazeneca; Drapier-Beche *et al.*, 1999).

Several hypotheses were suggested to explain the mechanism of epimerisation. The most common established theory claims the opening of the ring, the formation of an aldehyde acyclic intermediate (structure A in figure 3.1) and capturing of the aldehyde form by the hydroxyl OH at C5 to form the hemiacetal linkage (Silva *et al.*, 2006; Yamabe *et al.*, 1999; Capon, 1969).

Other theories suggested the formation of the aldehydrol structure B in figure 3.1 or the exchange of the hydroxyl group at C1 with a water molecule thus producing structure C in figure

3.1 (Capon, 1969). However, both possibilities were then excluded as it was proved by Ritternberg and colleagues (1958), that glucose undergoes oxygen exchange with water 30 times slower than the mutatrotation process. Therefore, the only possibility left is the formation of the aldehyde intermediate and re-forming of the ring by the hydroxyl OH at C5. Another possibility has been raised the cornerstone of which is capturing the aldehyde form at C4 instead of C5, thus forming the furnaose form; yet, the thermodynamic stability of the furnose form of glucose is very low and thus if the furnose form was accidently formed, it would rapidly undergo a ring opening to give the aldehyde form back again (Capon, 1969).

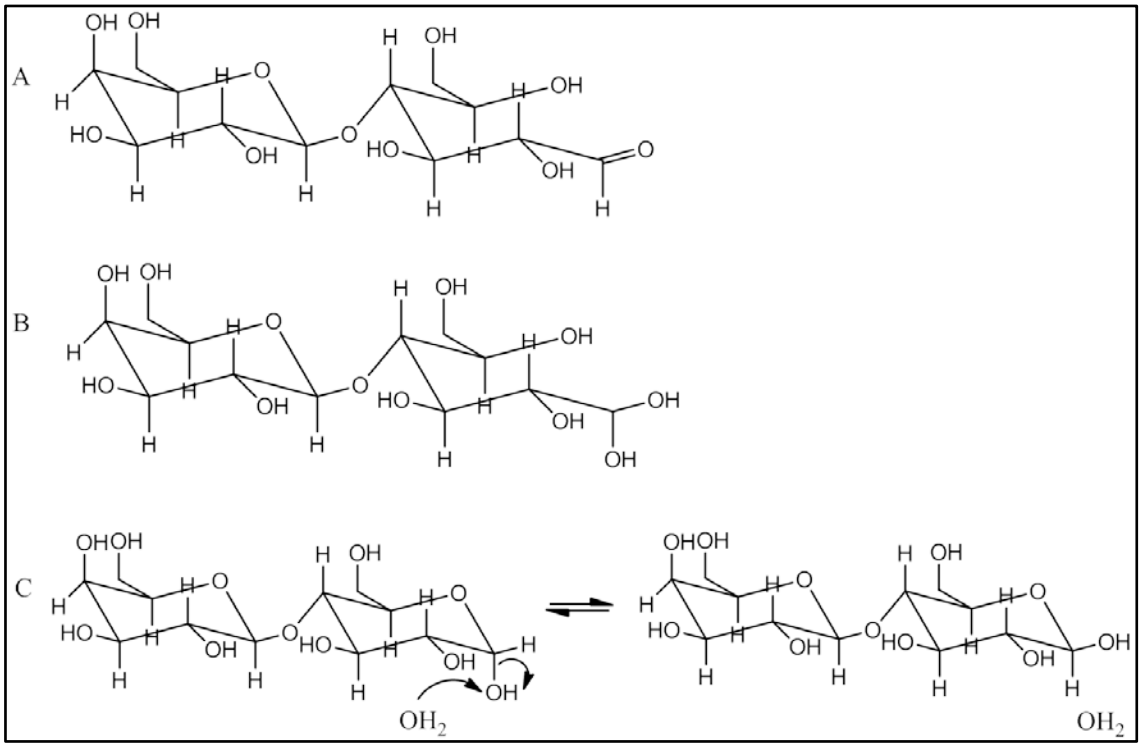

Figure 3.1 The intermediate formed during the epimerisation mechanism of lactose A) aldehyde acyclic intermediate; B) formation of the aldehydrol; C) exchange of the hydroxyl group at C1 with a water molecule.

The mechanism of epimerisation involves the formation of an aldehyde intermediate form separated by the α- and β- ring by two transition states which are TS1 and TS2 (Yamabe *et al.*, 1999). Yamabe *et al.* (1999) reported a theoretical study of the geometries of the epimerisation mechanism in two conditions (with no water present and in the presence of water molecules).

The proposed mechanism involved moving the proton to the ether oxygen forming a transition state denoted by TS1. TS1 can be defined as a four centred transition state which has a proton movement character and a small hydrogen bond angle of 125.3°. Then the aldehyde is formed followed by the closure of the ring by passing via the transition state TS2 which has the same geometry as TS1. TS1 and TS2 are two symmetric transition states which possess two activation energies E_a and $E_a^{'}$ with values of 125.5 kJ/mole and 84 kJ/mole respectively (Lefort *et al.*, 2006; Yamabe *et al.*, 1999). Both TS1 and TS2 are characterised by a large strain in their geometries when no water is present as the angles of the hydrogen bonds with the α- form were 140.8° and 147.3° (too small for proton transfer). However, in the presence of one water molecule, the strain of the hydrogen bond networks was relaxed and the isomerisation proceeded more uniformly. This is because the water molecule caused slight distortion of the pyranose form skeleton towards the aldehyde form. Thus, the hydrogen bond angles became 150.7° and 153.1°. It was also reported that adding two molecules of water produced angles of 167.0°, 161.5° and 170.4° and thus less strained structures (Yamabe *et al.*, 1999).

The epimerisation of lactose derives its high importance from its significant impact on the crystallisation process of lactose and on producing different anomeric ratios of α- and β-lactose (Ouiazzane *et al.*, 2008; Twieg *et al.*, 1968; Brinkman, 1976). These anomers, as discussed in chapters 1 & 2, have different physical properties which can affect any formulation that contains lactose.

Lactose crystallisation, including crystal formation and growth, can be influenced by several factors including the development of the nuclei, transport of the medium to the growing nuclei and the adsorption and orientation on the surface, which in turn, can be enhanced or suppressed by lactose epimerisation (Haase *et al.*, 1966). This strongly justifies the stance of numerous laboratories which consider epimerisation as a rate governing factor for crystallisation (Nickerson, 1962; Whitaker, 1933).

The epimerisation of lactose can be influenced by various factors such as temperature and concentration (Haase *et al.*, 1966). Garnier *et al.* (2002) reported that epimerisation of lactose can also occur upon heating of crystalline lactose in the solid-state. Le Barc'H and colleagues (2001) documented that increased temperature enhanced the epimerisation rate of D-glucose in aqueous solution. The addition of other molecules, such as sucrose, has also been reported to influence the epimerisation rate of lactose (Peter, 1928). Patel and co-workers (1970) proposed that the addition of sucrose (up to 40%) can slightly decrease the epimerisation rate of lactose. However, an increase in the sucrose content of more than 40%, can greatly decrease the epimerisation rate.

The investigation of epimerisation kinetics at different elevated temperatures was deemed fundamental to understand the epimerisation of lactose under conditions comparable to those met in the chocolate crumb manufacture.

The aim of this chapter is to explore the impact of temperature on the equilibrium and rate constants of epimerisation. To achieve this target, the following objectives were set:

1. Conducting polarimetry on aqueous solutions of lactose at different temperatures: 25 °C, 45 °C and 60 °C.
2. The determination of rate constants of epimerisation (overall, forward and reverse) and to evaluate the equilibrium constant as a function of temperature.

3.2 Materials and methods

The materials used in this chapter include: Sucrose, D (+) Saccharose, batch # 1084645 Fisher Scientific; software: Total commander 5.51 and APL Pro-data convertor.

The details of the following materials and equipments can be examined by referring to chapter 2: α-lactose monohydrate, β-lactose, HPLC water, PerkinElmer polarimeter 343, 10 cm pathlength cuvette, Comark C9003 thermometer, Freeze dryer, Bruker NMR 400, DMSO.

3.2.1 Sample preparation procedure (PerkinElmer polarimeter)

Lactose solutions (4% w/v) were prepared by weighing 0.4 g of α-lactose monohydrate (where the actual weight of the α-anomer is 0.384 g corresponding to 96% α-lactose content as confirmed by NMR in chapter 2) into a 10 mL volumetric flask and making up to volume with HPLC water. The samples were prepared at room temperature (measured as 25 °C ± 2). The zero time point was recorded as the initial time of mixing i.e. when the first drop of water touched the lactose powder. The α-lactose monohydrate solution was mixed for 3 min to ensure complete dissolution of the lactose powder and to obtain a clear lactose solution. A 4% w/v lactose concentration was used as the solubility of lactose is 7 g/100 mL (Fox *et al.*, 1998). Each solution was prepared and measured in triplicate at room temperature (25 °C ± 2). The optical rotation readings were recorded over a period of 4 h and the final measurement was recorded the following day of the experiment.

3.2.2 Instrument Preparation (PerkinElmer polarimeter 343)

A PerkinElmer polarimeter 343 supplied with a light source of sodium lamp (λ= 589 nm) and a 10 cm sample flow cell was employed. As most polarimeters are equipped with sodium lamps as a light source, the most commonly used wavelength is λ = 589. Therefore, all calibrations and sample measurements were performed at this particular wavelength. The polarimeter was prepared for calibration by zeroing the baseline for air and water. This was accomplished by taking a measurement when the flow cell was empty, then taking another measurement when the flow cell was full of water then subtracting these values from the baselines. Thereafter, the polarimeter was calibrated by a 1% w/v sucrose solution, measured in triplicates. The specific optical rotation measured is dependent on the wavelength selected (Table 3.1). The readings obtained were then compared to literature values where a correction factor was calculated (Hart *et al.*, 2011; Haus, 2010; Bergethon *et al.*, 1998). The correction factor will be presented and discussed in the next section.

Table 3.1 A presentation of the specific rotation of sucrose using different light sources and consequently different wavelengths (Haus, 2010).

Element (Light source)	Wavelength (nm)	$\alpha°/10$ cm.g.cm^{-3}
Li	670.8	50.51
Cd	643.8	55.04
Zn	636.2	56.51
Na	589.3	66.45
Hg	546.1	78.16
Cu	515.3	88.68
Cd	480.0	103.07
Cd	467.8	109.69
Hg	435.8	128.49
Fe	419.1	140.0
Fe	388.9	166.7
Fe	382.6	173.1

The temperature was kept constant at room temperature (25 °C ± 2) by surrounding the 10 cm sample flow cell by a water bath (25 °C) where water was circulated around the sample cell and a continuous record of temperature was maintained by connecting a Comark C9003 thermometer to the sample. All experiments were run following the same protocol of controlling and monitoring temperature of the sample and water bath to avoid any variations that might arise due to temperature fluctuations.

3.2.3 Instrument and sample preparation (Chirascan)

A protocol was established for sample preparation prior to running the sample on Chirascan: applied photophysics Chirascan CD/ORD/LD PMT supplied with a mercury lamp and detector 4005L038. The Chirascan was essential for the temperature controlled experiments as the Chirascan is equipped with a temperature probe which ensures a rapid and precise control of temperature with a regulation precision up to ± 0.02 °C. In addition, there is a built-in cuvette temperature probe that monitors sample temperature.

This protocol involved the following steps with the chronological order detailed below:

1. An empty 2mm cuvette cell was placed in the Chirascan instrument and the thermometer probe was inserted inside the cell.

2. The thermostat of the Chirascan was set at the desired temperature. Moreover, a Comark C9003 thermometer was connected to the cuvette to measure the actual temperature of the lactose solution inside the cuvette.
3. 10 mL of HPLC water, placed in an ice box at 4 °C, were pipetted into the glass vial containing lactose. This was done to slow the mutarotation process at the early stages of mixing (Lefort *et al.*, 2006). The solution was mixed for 2.5 min to ensure complete dissolution.
4. 300 uL solution samples were pipetted into the cuvette with a total time for mixing and heating up to ~ 3 min.

The kinetics of epimerisation of the prepared lactose solutions were investigated at different temperatures: 25 °C, 45 °C and 60 °C.

An initial method development was needed to establish the experimental parameters of the Chirascan. The experimental parameters involved the sample cell pathlength, the wavelength and the concentration of solution to be measured.

The concentration of lactose solution to be used was chosen based on the solubility value of lactose (7 g/100 mL). Therefore, the solution was prepared at a concentration of 4 g/100 mL to ensure fast yet complete dissolving of the solution in shortest time interval.

The wavelength considered for measurement of the 4% lactose solution was determined by measuring a spectrum between 600 nm and 200 nm using a 1 cm pathlength and at a 2 cm slit bandwidth (Figure 3.2). As the Chirascan is supplied with a mercury lamp, the wavelength corresponding to mercury lamp (λ = 313 nm) was observed in the run spectrum. It is important to note that a wavelength λ = 313 nm was used as it is the wavelength of a mercury lamp light source, thus the work can be easily reproduced on an ordinary polarimeter if a Chirascan is not available.

The spectrum showed that the optical rotation dispersion (ORD) recorded at 313 nm was higher than that of 589 nm; whereas, at 589 nm, the signal was very small and consequently it fell beyond the limits of detection of the Chirascan.

All of the optical rotation values reported in literature were measured at λ = 589 nm corresponding to the emission spectra of sodium (Hart *et al.*, 2011; Haus, 2010; Bergethon *et al.*, 1998). This is because the most commonly used polarimeters employ sodium lamp as their light source.

The optical rotation of 4% w/v lactose solution measured on the Chirascan at 589 nm was found to be 165° while the optical rotation signal value at 313 nm (the wavelength of the mercury lamp) was found to be 750° (Figure 3.2). A factor of 4.5 was produced by dividing 750 by 165°. Therefore, all the optical rotation values obtained at λ = 313 nm will be divided by a factor of 4.5 to produce an optical rotation value that is equivalent to values measured at λ = 589 nm and thus can be compared to values reported in literature (at the same wavelength). In other words, the optical rotation dispersion (ORD) curve indicates that a signal at 313 nm is 4.5 times bigger than a signal at 589 nm.

A 2 mm sample cell pathlength was chosen (instead of 1 or 10 cm- the most commonly or the standard pathlength used) to ensure a high thermal contact between the cuvette (containing the sample solution) and the temperature probe of the Chirascan.

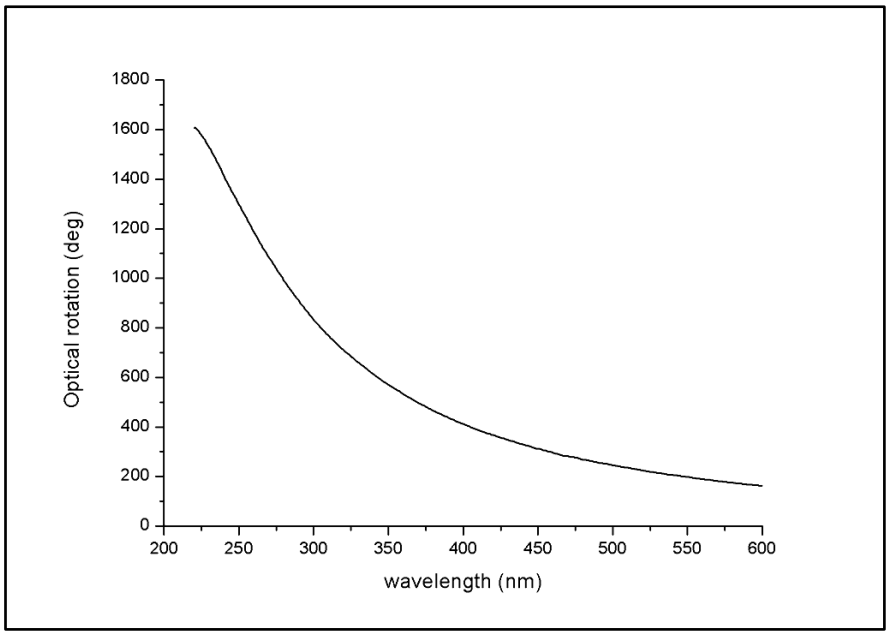

Figure 3.2 A spectrum (600 – 200 nm) of the optical rotation of 4% w/v α-lactose monohydrate solution at 2nm slit bandwidth and 1 cm pathlength.

The Chirascan instrument was first prepared for calibration by measuring air and water (as described in the previous chapter and section for the PerkinElmer polarimeter) then calibrated by a 1% w/v sucrose solution. The calibration of the instrument is a very important step to ensure that there is no drift in the baseline of the instrument and that it is working properly. Air was run through for 5 replicates at λ = 589 nm for 300 sec each time at 25 °C with a slit band width of 2 nm. This was followed by another 5 replicates of air at λ = 313 nm (which is the wavelength intended of use).

3.2.4 Freeze-drying of α-lactose monohydrate solution

This method is described in detail in chapter 2, section 2.2.3.

3.2.5 NMR of freeze-dried lactose

This sample preparation and the experimental parameters were described in chapter 2, section 2.2.8.

3.2.6 Purity of α-lactose monohydrate at 45 °C and 60 °C

To investigate whether the lactose solutions have suffered any potential chemical degradation (hydrolysis of lactose into its sugars: glucose and galactose) during the high temperature polarimetry experiments, ion chromatography (IC) technique was used for detection of the inversion products at low concentrations (Bernhardt *et al.*, 1999; Swallow *et al.*, 1991). As the concentration of inversion product was expected to be low, the sample preparation involved adding 1 mL of water to the 40 mg of the lactose material (in powder solid form) and then injecting the obtained solution into the IC. Therefore, solutions of lactose were stored at 45 °C and 60 °C over the length of the polarimetery experiments and subsequently freeze-dried to produce a cake solid form. The method of freeze-drying followed was described in detail in chapter 2. A 4% w/v lactose solution was used instead of 10% w/v solution, to mimic the concentration used in the Chirascan experiments. The preparation method involved storing the freshly prepared 4% w/v lactose solution for 4 h in two water baths set at 45 °C and 60 °C respectively. Both freeze-dried lactose batches were compared to the α-lactose monohydrate as received samples where an analysis of lactose for the sugar content including galactose and glucose was performed by Ion chromatography (IC).

3.3 Results

3.3.1 Optical rotation at 25 °C by PerkinElmer polarimeter

All of the optical rotation experiments using the PerkinElmer polarimeter were conducted in triplicate at 25 ± 2 °C. The specific rotation of α-lactose monohydrate was calculated using the observed optical rotation by employing the following equation:

$$[\alpha_{Obs}]_D = \frac{\quad}{\quad} \qquad \text{Equation 1}$$

Where $[\alpha_{Obs}]_D$ is the specific rotation in degrees at wavelength of sodium lamp light source $\lambda = 589$ nm, OR is the observed optical rotation, l is the pathlength in dm and C is the concentration in % w/v.

The specific rotation $[\alpha_{Obs}]_D$ of 4% w/v α-lactose monohydrate solution at 25 °C was calculated by extrapolating the exponential decay to the zero time point (Figure 3.3). The value obtained was 86° which agrees with the values reported in literature (Jawad *et al.*, 2012; Drapier-Beche *et al.*, 1999). It is worth noting that the extrapolation of the exponential decay was performed by two methods: manually and by fitting using Origin software (following equation

 where is the specific rotation reading at equilibirium) where both extrapolation methods produced the same results (Table 3.2).

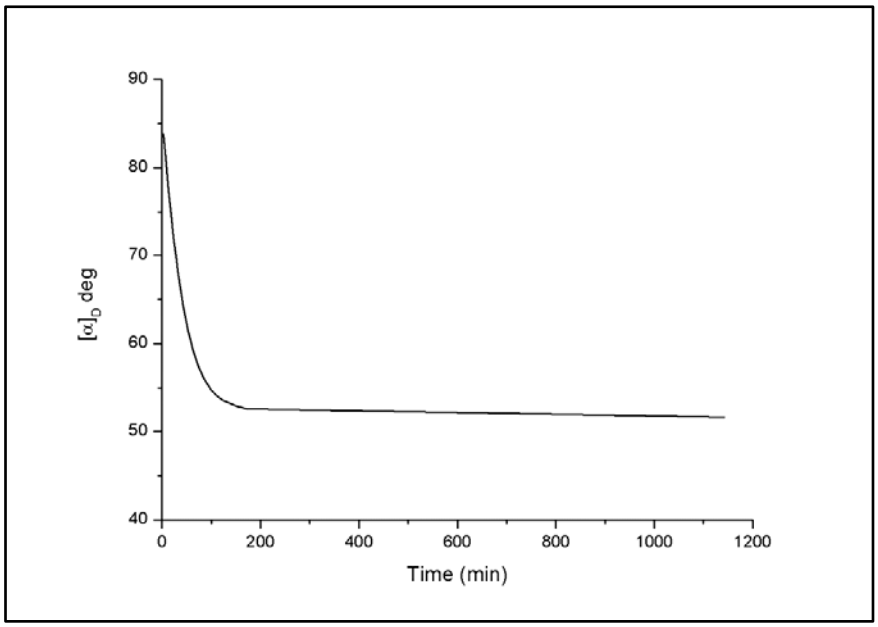

Figure 3.3 Mean optical rotation kinetics of 4% w/v α-lactose monohydrate at 25 °C using a PerkinElmer polarimeter (three replicates) (Examples of standard deviations: at t=30 min, SD= 4; t=100 min, SD= 3.6; t= 120 min, SD= 2.5).

The β- and α- anomeric ratio can be calculated as discussed in chapter 2 and according to Jawad *et al.* (2012).

For the initial time point, t = 0, 4% w/v solution;

$$83.2° = (0.96 \times [\alpha_{\alpha MH}]°) + (0.04 \times [\alpha_{\beta}]°) \qquad \text{Equation 2}$$

At equilibrium, 4% w/v solution;

$$51.6° = (0.37 \times [\alpha_{\alpha MH}]°) + (0.63 \times [\alpha_{\beta}]°) \qquad \text{Equation 3}$$

The specific rotation of the sample depends on several factors including temperature and concentration (Haase, 1996). As the experimental conditions for this experiment are constant i.e. same concentration and same temperature, $[\alpha_{\alpha MH}]°$ (defined as the specific optical rotation of 100% pure α-lactose monohydrate that has suffered no mutarotation) and $[\alpha_{\beta}]°$ (defined as the specific optical rotation of 100% pure β-lactose that has suffered no mutarotation) are also constant, thus the two simultaneous equations 2 and 3 can be solved to determine the values for $[\alpha_{\alpha MH}]°$ and $[\alpha_{\beta}]°$. Solving both equations using the data obtained from PerkinElmer at 25 ° C generated a mean value for $[\alpha_{\alpha MH}]°$ of 87° ± 1.6 (± SD, n = 3) and $[\alpha_{\beta}]°$ of 30° ± 1.6 (± SD, n = 3).

The fractions of β- and α- anomers, and thus their concentration, can be calculated using the following equations:

$$f_{\alpha} = ([\alpha_{\beta}]° - [\alpha_{Obs}]_D)/([\alpha_{\beta}]° - [\alpha_{\alpha MH}]°) \qquad \text{Equation 4}$$

$$f_{\beta} = ([\alpha_{\alpha MH}]° - [\alpha_{Obs}]_D)/([\alpha_{\alpha MH}]° - [\alpha_{\beta}]°) \qquad \text{Equation 5}$$

Where $[\alpha_{Obs}]_D$ was defined as the observed specific optical rotation for any sample of lactose under study and as a measured experimental value therefore it was a known dependent variable.

The epimerisation mechanism of lactose follows reversible first order kinetics (Capon, 1969; Haase *et al.*, 1966).

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

$$\alpha\text{–Lactose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \beta\text{–Lactose}$$

Where k_1 is the forward rate constant and k_2 is the reverse rate constant.

The rate of the decomposition of the reactant A can be expressed as follows (Yon-Kahn *et al.*, 2010; Florence *et al.*, 2011)

Equation 6

Equation 7

Where [A] is the concentration of the reactant, in this case α-lactose monohydrate; [B] is the concentration of the product, in this case β-lactose; is the concentration of the reactant at initial time point.

Using equation 8 produces an equation that can be re-arranged to generate equation 9.

Equation 8

Equation 9

Where is the concentration of the reactant at time t; is the concentration of the product at equilibirium.

As k_1 and k_2 are constants, equation 9 can be integrated to obtain the following equation:

Equation 10

Therefore, a plot of ln $B_{eq}/(B_{eq} - B_t)$ versus time should produce a slope which equals the overall rate constant of the reaction; where B_{eq} represents the % w/v concentration of β-anomer at equilibrium and obtained by multiplying the fraction of β- anomer f_β at equilibrium by 100; B_t represents the % w/v concentration of β- anomer at time t and obtained by multiplying the fraction of β-anomer f_β at time t by 100.

When the reaction is reversible, equilibrium will be reached after a certain period of time (Yon-Kahn *et al.*, 2010). Once the equilibrium is reached, the equilibrium constant K can be calculated as the ratio of the forward and reverse rates.

A plot of ln $B_{eq}/(B_{eq} - B_t)$ versus time was constructed and produced a straight line with a correlation coefficient of $r^2 = 0.9994$ (Figure 3.4). This proves that the epimerisation kinetics of α-lactose monohydrate fits the reversible first order rate equation. The slope obtained equals $4.005 * 10^{-4} \pm 0.0$ (n = 3 ± SD). Therefore, the overall rate constant of the epimerisation kinetics of α-lactose monohydrate at 25 °C is 0.0004 s^{-1}.

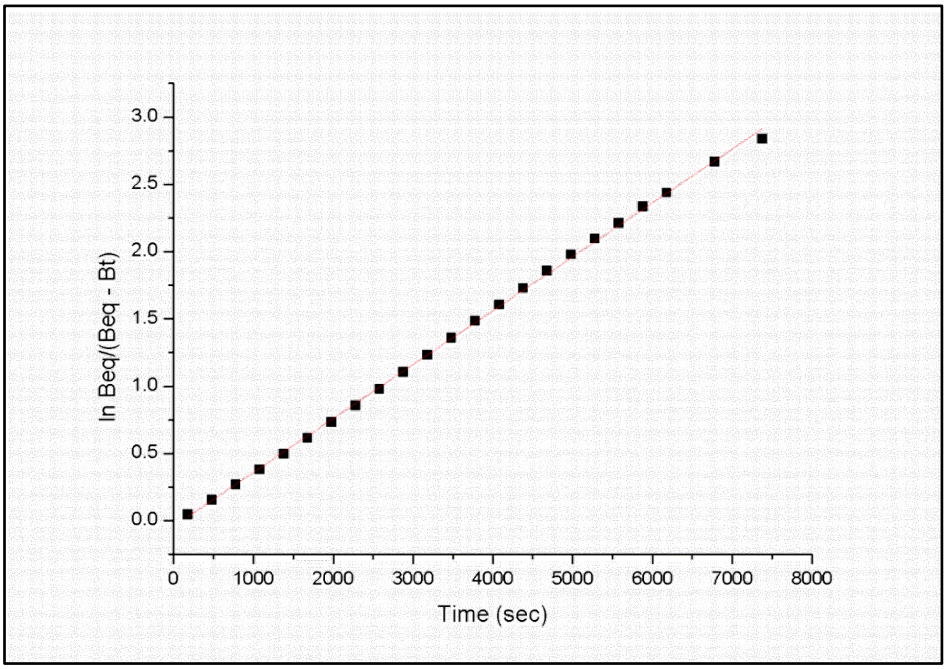

Figure 3.4 A plot of ln $B_{eq}/(B_{eq} - B_t)$ versus time of α-lactose monohydrate at 25 °C with a mean overall rate constant of $4.005 * 10^{-4} \pm 0.0$ s^{-1} (n = 3 ± SD).

The forward and reverse rate constants were calculated using both the overall rate constant and the equilibrium constant K and the values obtained are reported in Table 3.2.

3.3.2 Optical rotation of α-lactose monohydrate at 25 °C by Chirascan

The Chirascan was calibrated at both wavelengths: 589 nm and 313 nm. As discussed above, all of the measurements were taken at 313 nm (wavelength referring to the mercury lamp), as it produces a higher optical rotation value than that produced at 589 nm. This is clearly shown in the optical rotation spectrum of lactose in figure 3.2. However, data with reference to the D-line of sodium lamp (λ = 589 nm) are the most frequently published yet these data do not

present values measured at the maxima of the ORD curves (Gergely, 1989). Therefore, the calibration was performed at the wavelength of measurements (λ = 313 nm) and the wavelength mostly reported in literature (λ = 589 nm) for comparison. The data presented in the figures and tables below were measured at 313 nm and then divided by the correction factor. α-lactose monohydrate was run over time at 25 °C in 3 replicates and the mean value was plotted in figure 3.5.

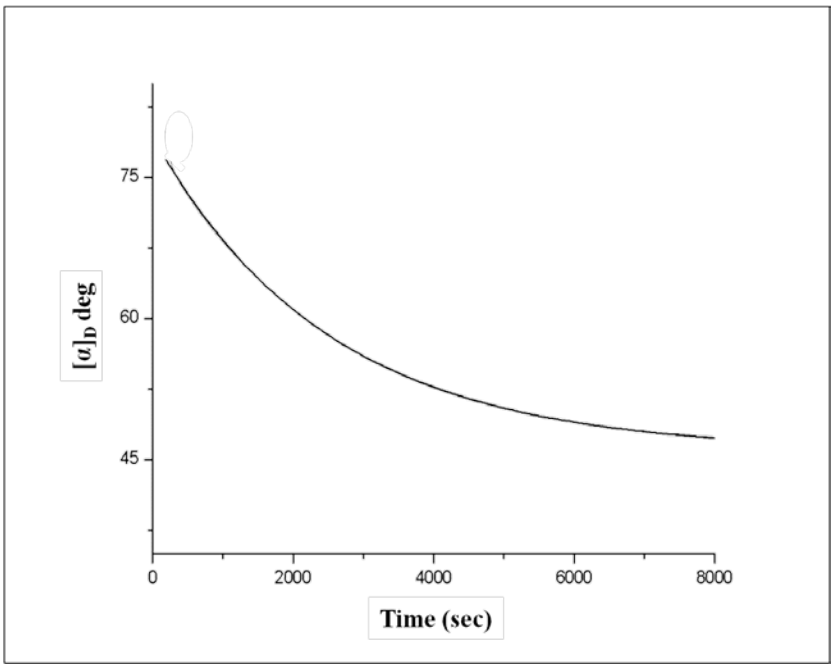

Figure 3.5 Mean optical rotation kinetics of 4% w/v α-lactose monohydrate at 25 °C using a Chirascan (three replicates) (Examples of standrard deviations, at t=1800 sec, SD= 1.23; t=3600 sec, SD= 1.16; t=7200 sec, SD= 1.18).

The exponential decay curves (generated from both PerkinElmer polarimeter and x) were extrapolated to determine the initial specific rotation of the solution at the zero time point. The extrapolation was done using two methods: manual extrapolation and fitting extrapolation following equation (Origin software) where is the specific rotation reading at equilibrium. The produced data are listed in table 3.2. Both methods produced similar results with slight variations. However, it was decided to rely on the manual extrapolation data as it produced more accurate $[\alpha_{Obs}]_D$ onsets. Moreover, manual extrapolation is usually the classical

way of extrapolating such exponential decays. Therefore, all of the data reported in this chapter were extrapolated manually.

As discussed in the previous section, the epimerisation kinetics of lactose fits a reversible first order kinetics. Consequently, a plot of ln $B_{eq}/(B_{eq} - B_t)$ versus time produced a straight line with a slope, and thus an overall rate constant of $4.08 * 10^{-4}$ sec^{-1} ± 0.15 (n = 3, ±SD).

Table 3.2 A summary of the specific rotations $[\alpha_{Obs}]_D$: the observed specific rotation at time = 0, as not at another time, $[\alpha_{\alpha MH}]°$ and $[\alpha_{\beta}]°$ determined using equations 4 and 5 and rate constants of α-lactose monohydrate at 25 °C by both PerkinElmer polarimeter and Chirascan. B_{eq} represents the % concentration of β- anomer at equilibrium and A_{eq} represents the % concentration of α- anomer at equilibrium. Both A_{eq} and B_{eq} were determined by calculating the f_{α} and f_{β} using equations 6 and 7 from chapter 2. The β/α ratio at equilibrium represents the ratio of B_{eq}/A_{eq}.

	Chirascan Manual Extrapolation			**Chirascan Fitting Extrapolation**			**PerkinElmer Manual=fitting**		
	R1	R2	R3	R1	R2	R3	R1	R2	R3
$[\alpha_{Obs}]_D$	78.5	80	77	79.0	81.0	78.1	83.2	85.5	86
$[\alpha_{\alpha MH}]°$	80.6	82.2	79.2	81.2	83.3	80.3	85.3	87.8	88.3
$[\alpha_{\beta}]°$	26.4	27.1	24.1	26.1	26.2	23.5	31.7	28.6	29.1
$[\alpha_{Obs}]_D$ at equilibrium	46.5	47.0	44.5	46.5	47.0	44.5	51.6	50.5	50.9
β/α ratio at equilibrium	62.9/37.1	63.9/36.1	62.9/37.1	62.9/37.1	62.9/37.1	63.1/36.9	62.9/37.1	63.9/36.1	62.9/37.1
$k = k_1 + k_2$	$4.21 *10^{-4}$	$4.10*10^{-4}$	$3.92*10^{-4}$	$4.21 *10^{-4}$	$4.10*10^{-4}$	$3.92*10^{-4}$	$4.00*10^{-4}$	$4.00*10^{-4}$	$4.00*10^{-4}$
r-squared	0.9983	0.9994	0.9999	0.9983	0.9994	0.9999	0.9956	0.9993	0.9993
$K = B_{eq}/A_{eq}$	1.69	1.76	1.7	1.7	1.74	1.7	1.69	1.69	1.7
k_1 (sec^{-1})	$2.65*10^{-4}$	$2.62*10^{-4}$	$2.47*10^{-4}$	$2.65*10^{-4}$	$2.62*10^{-4}$	$2.47*10^{-4}$	$2.52*10^{-4}$	$2.52*10^{-4}$	$2.52*10^{-4}$
k_2 (sec^{-1})	$1.56*10^{-4}$	$1.48*10^{-4}$	$1.45*10^{-4}$	$1.56*10^{-4}$	$1.48*10^{-4}$	$1.45*10^{-4}$	$1.48*10^{-4}$	$1.48*10^{-4}$	$1.48*10^{-4}$

The actual values of the observed $[\alpha_{Obs}]_D$ and calculated $[\alpha_{\alpha MH}]°$ at the initial time point were slightly different (Table 3.2). This can be explained in table 3.3 which reports a similar range

of variations among the $[\alpha_{Obs}]_D$ reported in literature. Thus, the slight variations in the data generated in this chapter are acceptable as they fit within the range reported in literature (Table 3.3).

Table 3.3 $[\alpha_{Obs}]_D$ values reported in literature at λ = 589 nm, where $[\alpha_{Obs}]_D$ is the observed specific rotation when a nearly pure sample of α-lactose monohydrate is added to water at t=0.

Reference	**$[\alpha_{Obs}]_D$ at t=0**	**$[\alpha_{Obs}]_D$ at Equilibrium**	**β:α ratio**
Drapier-Beche, 1999	+ 84°	-	-
Fox *et al.*, 2000	+ 89.4°	+ 55.4°	63:37
Hockett *et al.*, 1931	+ 90°	-	-
Walstra *et al.*, 2005	+ 91.1°	-	-
Tamime, 2006	+ 89.4°	-	-
Haase *et al.*, 1966	-	+ 55.23°	-
Jawad *et al.*, 2012	+ 82.6°	+ 50.6°	60:40
Hans-Dieter Belitz *et al.*, 2009	-	+ 53.6°	-

This relatively wide range in values for the $[\alpha_{Obs}]_D$ can be clearly understood if the ambiguity in the experimental details given in the literature are discussed. For example, most of the reported $[\alpha_{Obs}]_D$ for α- lactose monohydrate do not state the % anomeric purity of the sample (i.e. the α- and β- anomer content) (Tamime, 2006; Walstra *et al.*, 2005; Hans-Dieter Belitz *et al.*, 2009; Fox *et al.*, 2000; Drapier-Beche, 1999; Haase *et al.*, 1966; Hockett *et al.*, 1931). Moreover, the temperature at which the polarimetry experiments were conducted does vary slightly for some of the reports and in some cases it is not clearly stated at all. For example, Drapier-Beche (1999) states that the reported $[\alpha_{Obs}]_D$ values are measured at 20 °C and Jawad *et al.*(2012) reported the $[\alpha_{Obs}]_D$ values measured at 25 °C while the $[\alpha_{Obs}]_D$ values reported by Fox *et al.*(2000) does not make any reference to temperature. Another source for this variation is that the form of lactose used is not clearly stated in most of the literature whether they used α-lactose monohydrate or lactose anhydrous. For example, Drapier-Beche (1999) found a difference between the $[\alpha_{Obs}]_D$ of α-lactose monohydrate (84°) and the $[\alpha_{Obs}]_D$ of anhydrous α-lactose (88.8°). However, most of reports do not state this difference and it is generally referred to as α-lactose.

Therefore, the variance in the $[\alpha_{Obs}]_D$ reported in literature is a result of not stating or controlling the associated experimental parameters. This study makes a significant impact as the observed $[\alpha_{Obs}]_D$ values were associated with clearly stated experimental parameters and the lactose sample anomeric purity was clearly controlled and recorded.

It is also worth mentioning that the β/α ratio at equilibrium was determined by two investigators using two different methods. Fox *et al.* (2000) determined the ratio by polarimtery to be 63/37. Whereas, Jawad *et al.* (2012) determined the ratio by NMR to be 60/40.

The calculations of the overall rate constant k, forward rate constant k_1 and reverse rate constant k_2 (obtained from the manual fitting of the Chirascan results) have shown that the forward rate of mutarotation from α-lactose monohydrate to β-lactose ($k_1 = 2.58 * 10^{-4}$ $sec^{-1} \pm 0.09$) is higher than the reverse rate of mutarotation from β-lactose to α-lactose ($k_2 = 1.49 * 10^{-4}$ $sec^{-1} \pm 0.05$). The Chirascan software calculated the overall rate constant automatically by applying the exponential decay which produced an overall rate constant $k = 3.95 * 10^{-4}$ sec^{-1} which is very similar to the value generated using the method approached in this chapter, $k= 4.08 * 10^{-4}$ sec^{-1}. This gives high confidence in the Chirascan software analysis and thus can be applied further in this chapter, to calculate the overall rate constant.

3.3.3 Purity of α-lactose monohydrate at 45 °C and 60 °C

Prior to investigating the mutarotation kinetics of lactose at 45 °C and 60 °C, it was essential to determine any possible degradation upon exposure to such high temperatures for a long period of time (~ 4 h). Thus, the 4% w/v lactose samples were held at elevated temperatures as those investigated in the polarimeter and for the same length of time. The solutions were then freeze-dried to remove water and halt any further epimerisation or possible degradation. Next, they were stored in the stable solid form and analysed by IC. The investigated sugars are the monomers of lactose which are glucose and galactose and the results are listed in table 3.4.

Table 3.4 Analysis of lactose degradation products (glucose and galactose) at 45 °C and 60 °C by IC.

Lactose	Sugars	Content mg/kg of lactose sample	Content (%w/w)
Lactose as received (25 °C)	Glucose	123	0.0123
	Galactose	115	0.0115
Lactose (freeze dried 45 °C)	Glucose	135	0.0135
	Galactose	133	0.0133
Lactose (freeze dried 60 °C)	Glucose	148	0.0148
	Galactose	129	0.0129

The results obtained clearly showed that miminal degradation had occurred at high temperatures as the obtained values (expressed in mg) were very low compared to 1 kg of the sample. Therefore, all of the results obtained from the 45 °C and 60 °C experiment with any findings observed correspond to pure lactose rather than its degradation products.

3.3.4 NMR of freeze-dried lactose

The NMR of the freeze-dried samples prepared at all temperatures 25 °C, 45 °C and 60 °C show that all samples produced the same final equilibrium of β/α anomeric ratio (Table 3.5). Therefore, it can be concluded that the final equilibrium of the freeze-dried material is constant at the elevated temperatures up to 60 °C.

Table 3.5 The equilibrium β/α anomeric ratio of freeze-dried lactose samples at different temperatures, as determined by NMR, 3 replicates (one batch at each temperature was prepared and each batch was sampled 3 times on NMR).

Temperature	25 °C		45 °C		60 °C	
Anomeric composition of freeze-dried lactose (%)	β	α	β	α	β	α
	60.2	39.8	60.0	40.0	60.0	40.0
	60.3	39.7	60.1	39.9	60.2	39.8
	60.0	40.0	60.1	39.9	60.1	39.9
Mean (n=3)	60.2	39.8	60.1	39.9	60.1	39.9
SD	0.2	0.2	0.1	0.1	0.1	0.1

Haase *et al.* (1966) reported that at different temperatures, the optical rotation values at the equilibrium stage change, without calculating or reporting the corresponding β/α ratio. Therefore, the following step was to run the experiments of the lactose epimerisation at 45 °C and 60 °C to investigate the final equilibrium β/α ratio.

3.3.5 Optical rotation at 45 °C and 60 °C by Chirascan

Prior to investigating the epimerisation kinetics of α-lactose monohydrate at 45 °C and 60 °C, it was essential to confirm that the Chirascan is functioning properly and reproducibly on that temperature range. Thus, the instrument was calibrated at both temperatures. Air, water and 10% w/v sucrose solution were measured in triplicates at 25 °C, 45 °C and 60 °C on the same day. The results clearly show that temperature variations had no significant impact on the optical rotations of both air and water. However, the optical rotation of sucrose demonstrated a decreasing trend as the temperature increased.

Table 3.6 The optical rotation values of the calibration of Chirascan at 25 °C, 45 °C and 60 °C performed on the same day where 10% w/v sucrose aqueous solution (freshly prepared) was used. All samples were run in triplicates at a wavelength λ= 589 nm.

	Optical Rotation (n=3, ± SD)		
	25 °C	**45 °C**	**60 °C**
Sucrose	66.2 ± 0.02 deg	62.1 ± 0.05 deg	61.7 ± 0.06 deg
Air	- 9.0 ± 0.4 mdeg	-10.2 ± 0.15 mdeg	-9.3 ± 0.4 mdeg
Water	-16.0 ± 0.14 mdeg	-17.9 ± 0.36 mdeg	-17.8 ± 0.07 mdeg

The results listed in table 3.6 illustrated that the specific rotation of sucrose decreased with temperature. The underlying mechanisms and driving factors behind such variations are discussed in detail in the discussion section.

After the calibration of the Chirascan was accomplished, the epimerisation kinetics of α-lactose monohydrate were investigated at 45 °C. The specific rotation values of lactose $[\alpha]_D$ were

deduced from the generated optical rotation values by employing Equation 1. The calculated $[\alpha_{Obs}]_D$ was then plotted versus time (Figure 3.6). It is important to note that the Chirascan started recording the optical rotation readings after 280 sec from the zero time point of preparing the 4% w/v α-lactose monohydrate. The 280 sec include the time needed to dissolve the lactose solution plus the time needed to achieve the target temperature of the solution, which in this case is 45 °C.

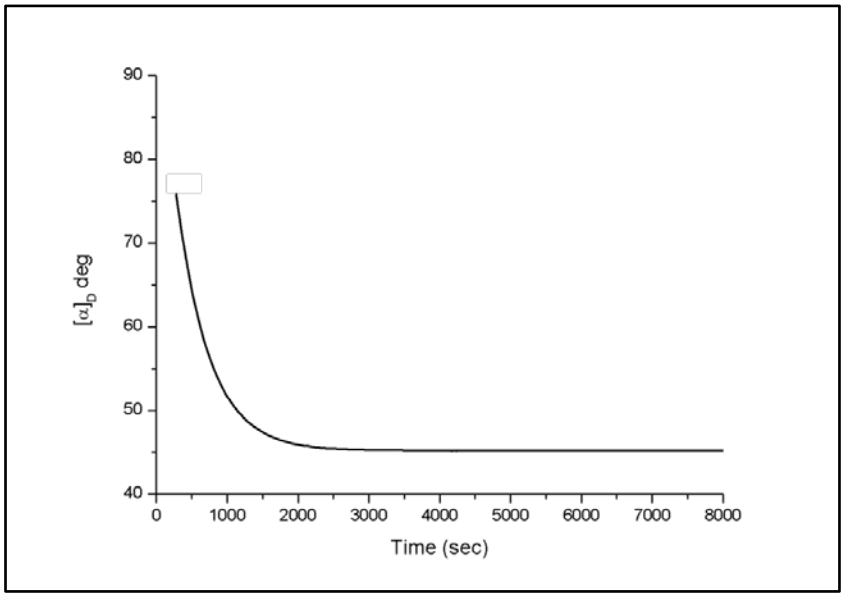

Figure 3.6 Mean specific optical rotation of 4 % w/v α-lactose monohydrate at 45 °C at wavelength λ= 589 nm, measured by Chirascan (three replicates).

Thereafter, the epimerisation kinetics of α-lactose monohydrate were investigated at 60 °C. The specific rotation values of lactose $[\alpha_{Obs}]_D$ were calculated using the optical rotation data according to Equation 1. The calculated $[\alpha_{Obs}]_D$ was then plot versus time (Figure 3.7).

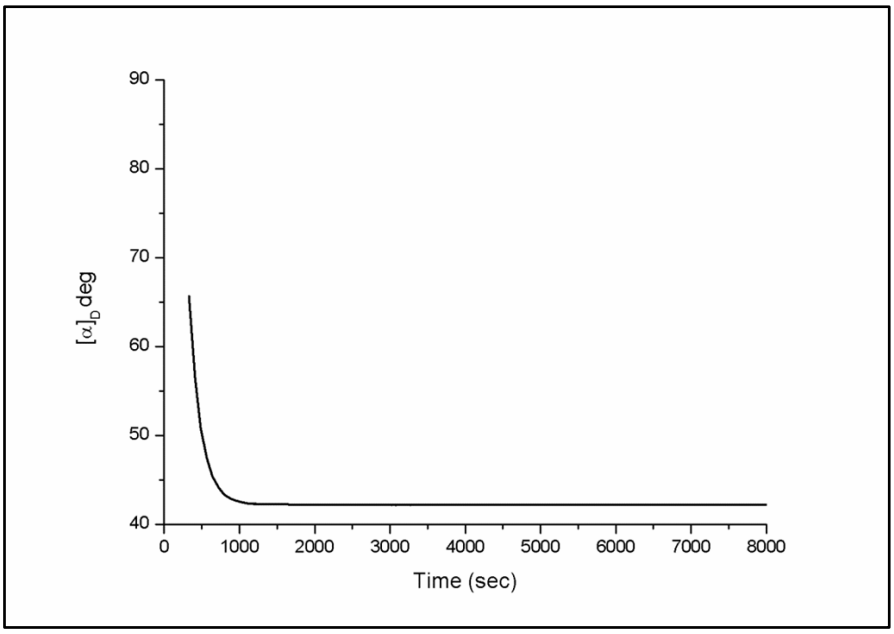

Figure 3.7 Mean specific optical rotation of 4 % w/v α-lactose monohydrate at 60 °C at wavelength λ= 589 nm, measured by Chirascan (three replicated).

The Chirascan software calculated the overall rate constant of the epimerisation of α-lactose monohydrate at 45 °C and 60 °C to be 21.53 * 10^{-4} and 35.01 * 10^{-4} sec^{-1} respectively. It was not possible to calculate the overall rate constant, forward rate constant and the reverse rate constant using the approach applied in this chapter for the 25 °C experiments. This is because, in the 25 °C experiments, the final equilibrium β/α ratio was already known (as determined by NMR in chapter 2; Jawad *et al.*, 2012). However, the final equilibrium β/α ratio is not known at 45 °C and 60 °C. Extensive literature search has been done to find whether the equilibrium ratio has been determined at 45 °C and 60 °C by other laboratories but no results were found. The equilibrium ratio of β/α determined by NMR of freeze-dried lactose at 45 °C and 60 °C were not used as a definitive guide for the final equilibrium as there is a concern that during freeze drying, the material tend to reach the final usual equilibrium i.e. β/α 60/40 even if the starting feed solution was of different final equilibrium β/α ratio.

Table 3.7 A summary of the $[\alpha_{Obs}]_D$ at initial time point and the $[\alpha]_D$ at equilibrium of freeze-dried lactose at 45 °C and 60 °C, 3 replicates (measured by Chirascan).

	45 °C				60 °C			
	R1	R2	R3	Mean ±SD	R1	R2	R3	Mean ±SD
$[\alpha_{Obs}]_D$	88°	88°	88.5°	88.2° ± 0.3	89°	92°	87.5°	89.5° ± 2.3
$[\alpha]_D$ at equilibirium	45.3°	44.7°	45.4°	45.1° ± 0.4	42.2°	42.0°	42.0°	42.1° ± 0.1

The determined rate constants from 45 °C and 60 °C indicate that the higher the temperature, the faster the equilibrium was achieved and the shorter the time needed for the run.

3.4 Discussion

Lactose epimerisation is a first-order reversible reaction that interconverts from the α-anomer to the β-anomer which both differ in the solubility, sweetness, refractive index, NMR spectra, and infrared spectra (Capon, 1969; Haase *et al.*, 1966). The investigation of epimerisation kinetics at different temperatures was essential to understand the epimerisation of lactose under conditions comparable to those met in the chocolate crumb manufacture and the spray-drying of lactose (which both involve exposure to high temperatures). Therefore, the aim of this chapter was to explore the impact of temperature on the equilibrium and rate constants of epimerisation of lactose. The objectives set to achieve the set target involved conducting polarimetry experiments of lactose at 25 °C, 45 °C and 60 °C and the determination of the rate constants (overall, forward and reverse) and the evaluation of the equilibrium constant as a function of temperature.

The epimerisation kinetics of 4% w/v α-lactose monohydrate were studied at 25 °C first using PE polarimeter. The attained results showed that the lactose epimerisation reaction has an overall rate constant k of $4.005 * 10^{-4}$ sec^{-1} ± 0.0 (n = 3, ± SD); forward rate constant k_1 of $2.51 * 10^{-4}$ sec^{-1} ± 0.0 (n = 3, ± SD) and a reverse rate constant k_2 of $1.48 * 10^{-4}$ sec^{-1} ± 0.0 (n = 3, ± SD). Therfore, the rate of epimerisation from the α-anomer to the β-anomer was faster than that

from the β-anomer to the α-anomer. This is a new finding in literature as the individual rate constants (forward and reverse) have not been previously determined. Haase and colleagues (1966) reported a detailed study about the epimerisation kinetics of α-lactose monohydrate at different temperatures and concentrations; where they calculated the overall rate constant k without assessing the forward k_1 and reverse k_2 rate constants.

The fact that the epimerisation from the α-anomer to the β-anomer was faster can be accounted for by the α-lactose structural properties, where the OH on the anomeric carbon suffered from steric effects which might increase the tendency of α-lactose to interact with water seeking a more relaxed structure (Jawad *et al.*, 2012).

The following step was to investigate the mutarotation kinetics of lactose at higher temperatures: 45 °C and 60 °C. However, the PerkinElmer polarimter did not possess a heating probe that could enable the conduction of the polarimetry experiments at elevated temperatures. Thus, the decision was to transfer the experiments to the Chirascan, which is an advanced polarimeter supplied with a temperature probe that enables polarimetry experiments at high temperatures, in this case, 45 °C and 60 °C. However, before starting with the high temperature experiments, it was essential to transfer the 25 °C experiments from the PE polarimeter to ensure the reproducibility in the results. Thus, the epimerisation kinetics of 4% w/v α-lactose monohydrate were studied at 25 °C then using a Chirascan. The results obtained revealed that the lactose epimerisation reaction has an overall rate constant k of $4.08 * 10^{-4}$ sec^{-1} ± 0.15 (n = 3, ± SD), forward rate constant k_1 of $2.51 * 10^{-4}$ sec^{-1} ± 0.1 (n = 3, ± SD), and a reverse rate constant of $1.45 * 10^{-4}$ sec^{-1} ± 0.06 (n = 3, ± SD). Thus, the similarity of the results produced by both Chirascan and PE polarimeter created a high confidence in the results produced by Chirascan and thus the 25 °C experiments were followed by the 45°C and 60 °C experiments.

Thereafter, the epimerisation kinetics of 4% w/v α-lactose solutions were analysed on the Chirascan at 45°C and 60 °C and the obtained results were analysed on the Chirascan software (Total commander 5.51. APL Pro-Data Converter) which showed an overall rate

constant of 21.53 * 10^{-4} sec^{-1} ± 1.1 (n = 3, ± SD) and 35.01 * 10^{-4} sec^{-1} ± 0.9 (n = 3, ± SD) respectively. This indicates that the higher the temperature, the faster the final equilibrium was reached and consequently the higher the value of the overall rate constant. Therefore, as the rate of the equation always increases with temperature, the Arrhenius equation can be applied to investigate the rate contant profile of the reaction.

Equation 11

Where k is the overall rate constant in sec^{-1}; A is a constant which represents the speed, size and orientation of the molecules; E_a is the activation energy, R is the universal gas constant, R = 8.31 J/mol.k; T is temperature in Kelvin.

The Arrhenius equation is usually applied to provide an insight into the nature of the reaction, the possible structures of the transition states and intermediates and the type of energy profile.

A plot of ln k vs (1/T) yielded a straight line of equation y = - 6292 x + 13.4 with a correlation coefficient r^2 of 0.9623. As E_a/R = - 6292, the activation energy E_a was calculated to be + 52.3 kJ/mole. The calculated activation energy (positive sign) is an endothermic energy which the glucose ring absorbs to be able to open its ring.

This is considered as a new finding as no previous experimental work measuring the activation energy of lactose epimerisation has been reported in literature. However, the activation energy of lactose epimerisation was determined based on computational analysis by Yamabe (1999) who used gaussian 94 software to study the geometries of the lactose molecule and subsequently predict the structure of two transition states. These transition states separate between the reactant and the product by an intermediate aldehyde structure form. The activation energies E_a and E_a' of both transition states were theortically determined to be 125.5 kJ/mol and 84 kJ/mol respectively.

An extrapolation of the straight line to the 4 and 0 °C temperaures showed that at 0 °C, ln k = - 9.92 i.e. the overall rate constant at 0 °C = $0.49 * 10^{-4}$ sec^{-1} (Table 3.8).

Table 3.8 Overall rate constants k (sec^{-1}) and half lives $t_{1/2}$ (min) of the lactose epimerisation reaction between 0 and 60 °C. Temperatures 25, 45 and 60 °C represent the actual experiments whereas 0 and 4 represented an extrapolation of the fitted linear plot that resulted from the Arrhenuis equation.

Experiment	Temperature (K)	Overall rate constant k (sec^{-1})	ln k	1/T	Half life (min) $t_{1/2}$ = ln 2/k
0 °C	273	$0.49 * 10^{-4}$	-9.92	0.00366	235.7
4 °C	277	$0.90 * 10^{-4}$	-9.31	0.00361	128.4
25 °C	298	$4.08 * 10^{-4}$	-7.82	0.00336	28.3
45 °C	318	$21.53 * 10^{-4}$	-6.14	0.00314	5.4
60 °C	333	$35.01 * 10^{-4}$	-5.65	0.00300	3.3

This will be highly beneficial to pharmaceutical formulators to determine how long a lactose solution needs to be left in the fridge to maintain a particular anomeric ratio or until the final equilibrium is reached.

Thereafter, the time taken for 1% of the reaction to reach equilibrium and 99% to equilibrium can be calculated. This is due to the fact that the NMR method used, can detect as low as ~ 1% in terms of amounts of α- and β- present. Therefore, it was important to know how long it would take for 1% degradation at fridge temperature (4 °C) and 99% to get to equilibrium. This can calculated as follows:

$$\ln[A_t]-\ln[A_o] = -kt \qquad \text{Equation 12}$$

$$\ln([A_o]/[A_t]) = kt \qquad \text{Equation 13}$$

The time taken is $$t = \ln([A_o]/[A_t]) *1/k \qquad \text{Equation 14}$$

For 1% degradation, ln (100/99) * 1/k = t

For 99% degradation, ln (100/1) * 1/k = t

By substituting the rate constants k values, it was found that it takes ~ 3.4 minutes for 1% degradation at 0 °C, thus even at very cold temperatures, the impact of epimerisation can be reduced by preparing the lactose solution in ice cold water in a space of 3 minutes or under, otherwise epimerisation will cause a detectable change in equilibrium before it gets into the instrument.

Moreover, it was found that it takes approximately 26 hours for 99% of the equilibrium to be reached at 0 °C; at 25 °C, it takes 3 h; at 45 °C, it takes 1 h and at 60 °C, it takes 22 min for 99% equilibrium to be reached.

A closer view of the measured rate constant could be explained using the following approach. The lactose disaccharide is formed through a linkage of the anomeric carbon of galactose to the OH of glucose through an O-glycosidic linkage bond. Glucose can exist in two chair conformations A and B where one is more stable than the other depending on the interaction energies. Through the glycosidic linkage bond, lactose can exist in three different conformations; chair A, chair B and boat conformation. However, the boat conformation is very unstable due to the high steric crowding. An interconversion between the chair A and chair B forms exists yet it does not affect the configuration of the anomeric carbon. This interconversion between conformers is a fast reversible process that can hardly be seen or measured.

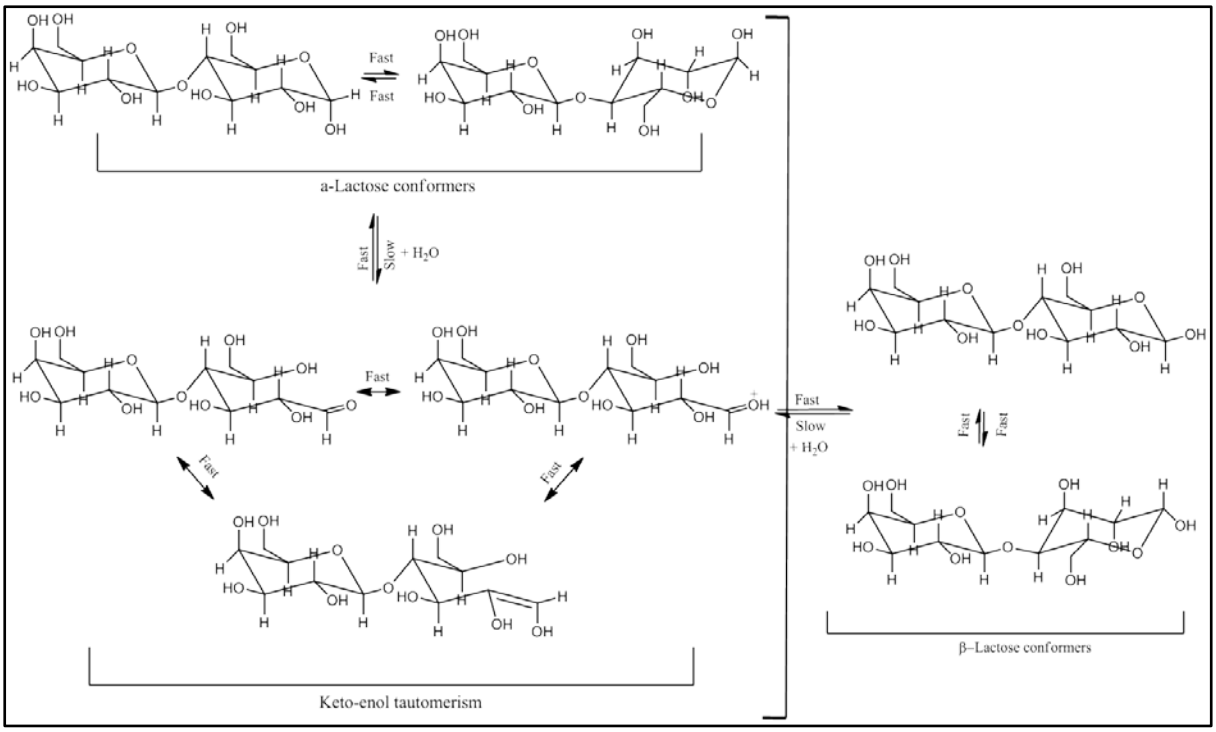

Figure 3.8 A schematic diagram of the possible conformations and intermediate structures of lactose during epimerisation.

Thereafter, lactose, in water, undergoes a ring opening to yield the aldehyde form which is captured by the hydroxyl groups at C5 to reform the hemiacetal linkage (Capon, 1969). Three processes are involved in the epimerisation mechanism i.e. formation of the aldehyde, addition of proton to the ring oxygen, breaking of the bond between C1 and the ring oxygen, and removal of the proton from the hydroxyl group at C1 (Capon, 1969). The process of the formation of the intermediate aldehyde is a slow process which might justify the postulate that the measured rate constant represents this step. Once the aldehyde structure is formed, keto-enol tautomerism occurs. Keto-enol tautomerism involves the movement of a proton and the shifting of bonding electrons. The keto form passes through an intermediate known as an enolate anion and is considered as a strong nucleophile. The keto-enol tautomers are not long lived structures and they do intercovert rapidly to the β-form. Therefore, it is believed that the measured rate constant does not represent this interconversion step. It can be concluded that the aldehyde and keto-enol groups must be very short lived as they are not detectable by spectroscopy.

Sucrose was also run on the Chirascan at different temperatures which showed a decreasing trend in the optical rotation values as the temperature increased. The optical rotation of sucrose decreased from 66.2° to 61.7 as temperature increased from 25 °C to 60 °C. Sucrose does not epimerise (Bhagavan, 2002). However, it has a flexible fructofuranose ring linked to the fairly rigid glucopyranose ring. This flexibility is enhanced by the presence of three primary OH groups where one of these hydroxyl groups is next to the glycosidic linkage (Mathlouthi *et al.*, 1995). Thus, sucrose can exhibit several different and well distinct conformations (Herve du Penhoat *et al.*, 1991). Steven *et al.* (1991) reported an equilibrium mixture of sucrose to have two linkages conformers, one similar to the crystalline structure and the other one possessing all the intramolecular hydrogen bonds. Therefore, these variations in optical rotation upon increasing temperature might be attributed to the conformational changes taking place at elevated temperatures.

3.5 Conclusion

It can be conluded that the rate constant of the epimerisation kinetics of lactose increases as temperature increases. The studies reported in this chapter proved that the rate of the epimerisation of α-latcose to β-lactose has dramatically increased as the temperature increased from 25 °C to 60 °C. This clear understanding of the epimerisation kinetics of lactose will have an impact on the control of its crystallisation behaviour.

Chapter four – Production and characterisation of a standard amorphous form of sucrose

The aim of this chapter is to report the development of a robust method for the production of amorphous sucrose standards which will be subsequently incorporated into model systems. Measurement of the chemical purity, with respect to hydrolysis and degradation, is an additional research objective of the work presented in this chapter.

4.1 Introduction

Sucrose constitutes up to 50% w/w of the chocolate composition (Beckett, 2000). It plays a vital role in the re-crystallisation of the chocolate crumb affecting both the flavour and the quality of chocolate (Beckett, 2000). There are several methods by which amorphous sucrose may be introduced into chocolate during the crumb manufacturing process. While producing the crumb, the concentrated milk, sugar and cocoa liquor mixture undergo drying under reduced pressure followed by a milling step. Both drying and milling have the potential to introduce a significant amount of amorphous sugar into the crumb (Bhugra *et al.*, 2007; Mathlouthi *et al.*, 1995; van Scoik *et al.*, 1990). In addition, amorphous sugars are highly hygroscopic, viscous with poor flowability and dispersibility (Chiou *et al.*, 2008). They possess greater pore size and volume; hence, they exhibit a greater sorption capacity compared with the crystalline state of the same material (Trivedi *et al.*, 2001). As a consequence, they tend to absorb water from their environment which could accelerate the degradation of chocolate particularly upon storage. The re-crystallisation of amorphous sucrose leads to loss of flavour, a decrease in viscosity and an increase in water activity which may cause the degradation of chocolate (Beckett, 2000). When re-crystallisation occurs, the closely packed sugar particles may agglomerate, especially in the presence of water, forming sugar aggregates which remain tightly held together even if the fat melts affecting the flow properties severely and thus the viscosity and the flavour of chocolate (Beckett, 2000).

To evaluate the crystal forms and phases of the sugars within both crumb and chocolate, amorphous sucrose standards need to be produced with a fully characterised composition.

Therefore, the purity of the standards and the investigation of the presence of potential chemical impurities; for example, invert sugars (glucose & fructose) in amorphous sucrose is considered a key factor in the thesis.

The most common methods used for the production of amorphous powders include quench cooling, spray-drying, freeze-drying and milling. Quench cooling cannot be applied to generate amorphous sucrose since heating a sucrose sample above its melting point will cause melt degradation and the formation of the brown-coloured product caramel. Lee and co-workers (2011) reported that melt-quenching is not recommended for the preparation of amorphous sugars as thermal decomposition chemically alters the sugar molecules and the subsequently formed amorphous matrix (sugar molecules plus decomposition components) may affect the physicochemical properties of the final product (Lee *et al.*, 2011).

The process of spray-drying has been widely used in both food and pharmaceutical manufacturing processes. Islam et *al.* (2010) documented the production of crystalline sucrose by spray-drying, where they proposed that the production of amorphous sucrose by spray-drying is limited by its low glass transition. Their investigation focused on determining the amount of sucrose crystallised during spray-drying using inlet temperatures that ranged between 95 - 220 °C. Hunter (2009) demonstrated that the production of sucrose glass by spray-drying using an inlet temperature of 165 °C and outlet 104 °C at a feed concentration of 17% w/v generates a sucrose glass with water content of 4 %. The same study also illustrated that reducing the feed concentration to 12.75% w/v and maintaining the inlet temperature at 165 °C was associated with an increase in water content of the sucrose glass to 8 %. The high water content generated by this approach renders spray-drying inapplicable for the present study because the water content should be kept considerably low. This is because the water content in both crumb and chocolate is of the order of 1 - 2 % w/w (Beckett, 2000).

It has been recently shown that the production of amorphous sucrose can also be attained by spray-drying a 10% w/v sucrose solution at a feed rate of 3.3 mL/min (Bhugra,

2007). The inlet temperature was controlled to maintain an outlet temperature of 62 °C. The amorphicity of the sucrose samples was confirmed using polarized light microscopy and X-ray powder diffraction. However, this approach did not consider the possibility of thermal degradation or hydrolysis of sucrose upon its exposure to high temperatures for relatively long time intervals. Tantalisingly, there has not been any report, even anecdotal, in the body of laboratory-based evidence accumulated in literature which highlights thermal degradation issues or the high water content of the amorphous powder generated by spray-drying methods.

Freeze-drying is another approach which has been reported to possess the capability to produce amorphous sucrose as illustrated by several independent investigations (van Scoik *et al.*, 1990; Kedward *et al.*, 1998; Chen *et al.*, 2001; Kawakami *et al.*, 2006; Alkhamis, 2009). These studies demonstrated that a high amorphous content of freeze-dried sucrose was produced as confirmed by various techniques e.g. powder X-ray diffraction (PXRD), differential scanning calorimetry (DSC) and Fourier transfer infrared spectroscopy (FT-IR). However, it has to be emphasised that the possibility of inversion of sucrose upon freeze-drying has not been investigated. As the whole freeze-drying process takes 5 days, it is essential to check whether any inversion is occurring in order to ensure that the freeze-dried product obtained is pure sucrose without any invert/isomeric sugar conversion.

Freeze-drying is selected as the method of choice to produce amorphous sucrose in this study. Every amorphous sucrose batch produced was checked for the presence of inversion products as these are formed by hydrolysis of sucrose. The hydrolysis products, glucose and fructose are called invert sugars (Figure 4.1). Inversion is an oxidation-reduction reaction that is considered as the initial reaction for most of the important reaction sequences of monosaccharides in food chemistry (Mathlouthi *et al.*, 1995).

Upon hydrolysis of sucrose (specific rotation $[\alpha]_D$ = +66º), a water molecule breaks the acetal bond in the sucrose structure forming an equimolar mixture of D-glucose ($[\alpha]_D$ = +52º) and D-Fructose ($[\alpha]_D$ = -92º) (Mathlouthi *et al.*, 1995). This happens by the attack of the hydrogen to the oxygen of the ether present between the two monosaccharides (glucose & fructose). The resulting equimolar mixture (Figure 4.1) exhibits a net negative rotation.

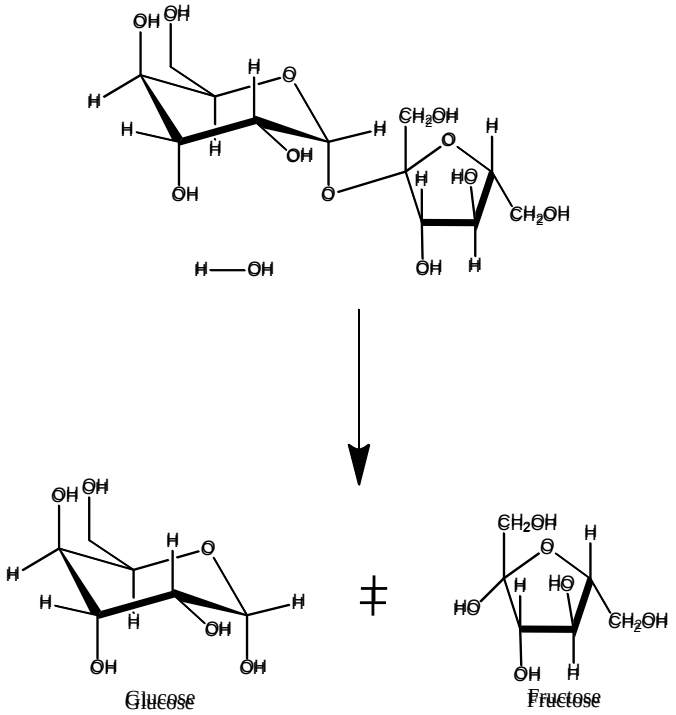

Figure 4.1 Inversion of sucrose to produce its invert sugars (glucose and fructose).

Hence, optical rotation can be used as a parameter to assess the degree of sucrose purity and evaluate invert sugar formation (Mathlouthi *et al.*, 1995). Optical rotation polarimetry will be the technique of choice used in this investigation to characterise such parameter. In brief, a polarimeter is an instrument which measures polarisability in solutions that contains molecules with chiral centres (optically active) thus rotating the plane of polarised light as the light passes through the sample (chapter 1).

The half life ($t_{1/2}$) of a sucrose inversion reaction, defined as the amount of time required for sucrose to invert from its 100% pure form to a 50% invert sugar in acidic medium, can be calculated using the following equation

$$t_{1/2} =$$ Equation 1

Where k is a temperature-dependent rate constant; k for sucrose at 25 °C is 0.208 h^{-1} (Asadi, 2007). The $t_{1/2}$ of sucrose inversion, in an acidic solution at 25 °C has been estimated to be 3.3 h. Several lines of investigation in sugar industry have reported that the $t_{1/2}$ of pure sucrose at 70 °C and pH 4.5 is 30 min (Asadi, 2007). Inversion is dependent on several factors and should consider where excess water is presented, e.g. in solutions prior to freeze-drying.

Invert sugars can play a role in suppressing crystallisation (Beckett, 2000). This justifies their inclusion in chocolate fillings and other candy formulations because they impede the crystallisation of the sucrose present (Beckett, 2000). Inverted sugars are primarily added to prevent crystallisation in marshmallows and balance the sweetness. It is noteworthy that the melting points of fructose and glucose are 126 °C and 157 °C respectively (Robert *et al.*, 2004).

As stated above, it is important to monitor and measure the presence of any invert sugars not only to avoid the impact they might have on the crystallisation profile but also to ensure a better accuracy and sensitivity in the thermo-analytical measurements taken for the amorphous sucrose standards.

The aim of this study is to establish a reproducible method for producing amorphous sucrose and to ensure that the starting standard sucrose is pure and that is not hydrolysing upon freeze-drying. Therefore, the objectives include:

1. Determination of the purity of crystalline sucrose prior to its application in the amorphous sucrose standard preparation.
2. Production of amorphous sucrose. Two techniques will be evaluated to fulfil this objective: spray-drying and freeze-drying.
3. Determination of levels of purity of the produced amorphous sucrose by measuring the levels of invert sugars present in sucrose if any.

4.2 Materials and methods

The materials used in this set of experiments are: sucrose: Silverspoon sugar (The Silver Spoon Company, United Kingdom); sucrose (AR Grade), B/N 0954097 purchased from Fisher Scientific (Fisher Scientific, United Kingdom); D-(+)-glucose ≥99.5%, part # G8270, purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd., England); D-(-)-fructose ≥99%, part # F0127, purchased from Sigma (Sigma-Aldrich Company Ltd., England); hydrochloric acid solution 6M, part # 31087, purchased from Sigma (Sigma-Aldrich Company Ltd., England); PerkinElmer polarimeter 430 (PerkinElmer, United Kingdom); TGA PerkinElmer Pyris 6 thermogravimetric analysis TGA (PerkinElmer, United Kingdom); (Mettler DSC 823e) attached to Haake EK90/MT intracooler and 40uL Mettler Toledo hermetic aluminium DSC pans and lids, DSC 2090 equipped with a refrigerated cooling system (TA instruments, UK).

There are other materials that were used and their details were mentioned in chapter 2, section 2.2 are: HPLC water, Gilson pipette pipetman P5000, phosphorus pentoxide, Aldrich atmosbag tape-seal, glassware including 7 mL glass bottles BIJOU, volumetric flask 50mL, glass beaker, measuring cylinder, pasteur glass pipettes 230mm. The instrumentation also involved Varian Girovac model GVD4 freeze-dryer, Buchi spray-dryer and Niro spray-dryer, DSC Q20 TA Instruments, Aluminuim hermetic DSC pans and lids, DMA 8000 PerkinElmer, DMA metal pockets, PerkinElmer TGA, -80 °C freezer, dessicator.

4.2.1 Preparation of the feed solutions for spray- and freeze-drying

For both spray- and freeze-drying, a 10% w/v aqueous feed solution of sucrose using crystalline material was prepared in HPLC grade water, this is a typical concentration used for these drying procedures.

4.2.2 Spray-drying

The 10% w/v feed solution was prepared exactly as described in section 4.2.1 and spray-drying was performed on the same day to prevent any degradation. The spray-dryer was operated

by switching on the aspirator at 95%, holding the inlet temperature at 150 ºC (Bhandari *et al.*, 1997). The flow rate of air was set at 400 L/min (Tang, 2008). Once the actual inlet temperature rises up to the pre-set value, the spray-dryer was rinsed out with water and left for 20 min to equilibrate. The feed sample was then placed in the spray-dryer to commence spray-drying the 10% w/w sucrose solution. Once the solution started feeding into the system, the atomiser air-flow was kept constant until all the feed solution was spray-dried. The outlet temperature of 65 ºC was monitored every 10 minutes to ensure that the system was at equilibrium. After the feed solution had all been spray-dried, the atomiser airflow rate and aspirator rate were increased to 100 % for 5 minutes to encourage the deposition of the product in the collecting cyclone.

4.2.3 Freeze-drying

Sucrose was freeze-dried using varian Girovac model GVD4 Freeze dryer. A 10% w/v sucrose aqueous solution was frozen at - 80 °C for 2 h then subjected to primary drying at – 50 °C for 72 h followed by secondary drying over phosphorous pentoxide P_2O_5. The vials were sealed under nitrogen atmosphere and stored in a dessicator over P_2O_5 in a controlled room temperature at 25 °C.

4.2.4 Thermogravimtric analysis (TGA)

PerkinElmer Pyris 6 thermogravimetric analysis TGA was calibrated for temperature and weight according to the TGA manual supplied by PerkinElmer. Samples of 5 - 10 mg of sucrose were loaded into an open pan using a micro spatula and heated at a heating rate of 10 ºC/min over a temperature range of 25 ºC to 150 ºC, with the sample mass measured as a function of temperature and time.

4.2.5 Determination of the purity of crystalline sucrose by ion chromatography (IC)

Ion chromatography is a chromatographic technique that separates ions based on their charge. IC is a fast, sensitive and selective tool that provides simultaneous detection. Thus, it was

selected to analyse glucose and fructose in the crystalline sucrose samples. Sucrose, glucose and fructose do not possess any chromophores; thus, detection using ultra violet (UV) spectrometry was excluded as post column derivatisation was needed. Sucrose and glucose become anionic at high pH (sucrose hardly inverts in alkaline media, Asadi, 2007) and can be separated by anion chromatography with a dilute potassium hydroxide (KOH) or sodium hydroxide (NaOH) eluent (Fritz *et al.*, 2008). Dionex ion chromatography system (ICS) 3000 was used and it was comprised of a Carbopack column attached to a Dionex Chromeleon version 6.80 as recorder. The mobile phase was milli-Q water (high pure distilled water) and 300 mM NaOH with a flow rate of 1 mL/min. The sucrose sample solutions were prepared by weighing approximately 0.1 g material and diluted to volume in 100 mL with milli-Q water. The mixed standards were prepared in the same manner as the sucrose solutions each containing 0.1g of sucrose, fructose and glucose. Both solutions were further diluted 10 fold and 10μL of each solution was injected for analysis.

4.2.6 Hot stage microscopy

A hot stage microscope was used to explore the physical transitions of both crystalline and amorphous sucrose. The hot stage microscope used was Leitz Dialux 22EB fitted with cross polariser filters attached to a camera (Q-Imaging QICAM fast 1934). In heating experiments, a hotstage (Linkham HSF 91) coupled to the microscope was used and the temperature and rate of heating of the sample were controlled by Linksys 32. Fisher sucrose and Silverspoon sugar were analysed by spreading a few crystals of each sugar separately onto the slide. The temperature profile involved a heating rate of 2 °C/ min from 25 °C to 220°C while images were recorded.

4.2.7 Optical rotation

The PerkinElmer polarimeter 430 was first zeroed for air and water and then calibrated for sucrose by measuring the optical rotation of a 10 g/mL of aqueous SigmaUltra sucrose solution, calculating its specific rotation and comparing it to reference values reported in the literature. Once the proper functioning of the polarimeter was confirmed, it was then zeroed by

a blank cell, filled with HPLC water. The samples were then measured according to table 4.1. The test procedure involved the preparation of a concentration of 10 % w/v of different sucrose/invert sugar compositions (Table 4.1).

Table 4.1 A plan of the Silverspoon sugar compositions tested in the polarimeter.

Weights dissolved in HPLC water in 50 mL flask	**Sample composition ratio**
20 g of sucrose	100:0 sucrose: invert sugar
15g sucrose: 2.5g fructose:2.5g glucose	75:25 sucrose: invert sugar
10g sucrose: 5g fructose: 5g glucose	50:50 sucrose: invert sugar
5g sucrose: 7.5g fructose: 2.5g glucose	25:50 sucrose: invert sugar
10g fructose:10g glucose	0:100 sucrose: invert sugar
20g of sucrose in 3 mL 6M HCL	Hydrolysed sucrose

Thereafter, three replicates of freeze-dried sucrose samples were measured to detect the presence of any invert sugar in freeze-dried sucrose.

4.2.8 Differential scanning calorimetry (DSC)

The TA Q20 DSC was calibrated while operating the instrument in the calibration mode and applying a heating rate of 10 °C/min and the default nitrogen purge gas parameters (50 mL/min). The baseline calibration was measured and corrected for the heat-flow deviation from zero of the DSC cell by running an empty cell through the chosen operating temperature range. The cell constant and onset slope were calculated by heating up indium pin-holed pan against a reference pin-holed empty pan at 10 ºC/min. Thereafter, the calibration was analysed according to a standard operating procedure SOP 2002/004 manual set by KCL labs. The literature values of the melting point of indium and the standard heat are 156.6 ºC and 28.71 J/g respectively. The cell constant was then calculated as the ratio of the measured enthalpy of fusion of indium and the literature value (Zeng, 1997). Additional DSC runs were made on an instrument equipped with a robot (Mettler DSC 823e) attached to Haake EK90/MT intracooler where dry N_2 was used as a purging gas, and 40uL Mettler Toledo hermetic aluminium DSC pans and lids.

The sample was prepared by weighing 3 - 5 mg of the produced sugar into a DSC pan, a pin-holed lid (to allow evaporation of any moisture embedded in the sample) was placed on the top of the pan and both were crimped to ensure that the pan was completely sealed. The DSC experiments were performed by equilibrating the system at 25 ºC, setting an isothermal mode for 10 minutes at 30 ºC followed by a ramping rate of 10 °C/min from 25 ºC to 200 ºC. The resulting data were analysed by the universal analysis software 2000 (TA Instruments).

4.2.9 Dynamic mechanical analysis (DMA)

The DMA instrument was calibrated for the force component by following the manufacturer's manual (Perkin Elmer). This was performed by tightening the stationary and the drive shaft sides of the cantilevers (with no pocket inside) and measuring the applied force. The temperature component was calibrated by indium with a melting point of 156.6 ºC and comparing the obtained value to the literature value.

The DMA samples were prepared by weighing 20 - 50 mg and loading them into a clean metal pocket made of a sheet of stainless steel then the powder pocket was folded to form an angle of 60° between the inner face of the pocket. The pocket was then crimped to ensure that the powder was fully compressed with the folded pocket and that a thin sandwich of 0.4 mm was formed. The sample was then clamped into the DMA instrument as a rectangular cross section in a single cantilever bending mode. One end of the pocket was clamped to the stationary side and the other end was undergoing an oscillating bending force through the drive shaft. The DMA furnace, which encased the clamping area completely, was used to apply the temperature program. The temperature of the sample was recorded by a platinum resistor sensor that is located behind the middle of the pocket (Royall *et al.*, 2005).

The experimental parameters employed in this test were a dynamic displacement of 0.05 mm, a multi-frequency mode of 1, 10 and 30 Hz and heating rate of 2 °C/min from 25 °C to 200 °C.

4.3 Results

4.3.1 Purity of crystalline sucrose (DSC and IC)

This study involved using ion chromatography and DSC. The DSC data conducted on the Mettler instrument have been previously reported (Carrera 2010). Fisher and Silverspoon sucrose were both examined by DSC where the onset of melting for Fisher sucrose was found to be 186 °C ± 0.6 (± SD, n = 3) with a melting enthalpy of 120 J/g ± 1 (± SD, n = 3) (Figures 4.2 & 4.3). These results comply with the melting point range (186 °C - 191°C and 160 °C - 191°C) reported in literature (Hurtta, 2004; Okuno, 2003; Roos, 1993). However, preceding the melting peak of Fisher sucrose, a small endothermic peak with an enthalpy of 3.7 J/g ± 0.4 (± SD, n = 3) at 153.7 °C ± 0.09 (± SD, n = 3) was observed. The Silverspoon sugar was also analysed in 6 replicates and showed an onset melting peak at 189 °C ± 0.3 (± SD, n = 6) with an enthalpy of 131 J/g ± 1.5 (± SD, n = 6) but no sign of any endothermic peak at 153°C was observed.

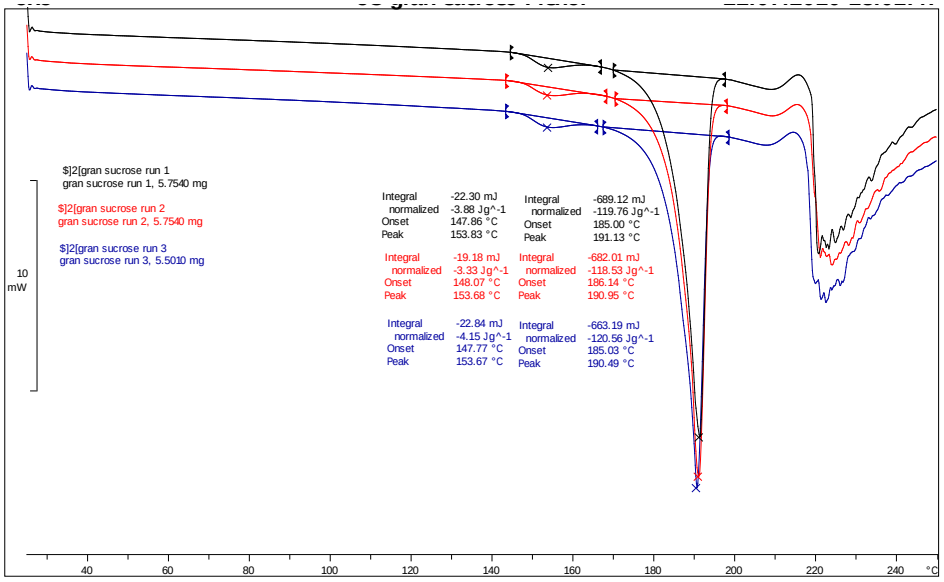

Figure 4.2 DSC Curve of crystalline Fisher sucrose (triplicate) (Carrera, 2010), showing that the melting peak of Fisher sucrose is preceded by a small endothermic peak at 153.7 °C ± 0.09 (± SD, n = 3).

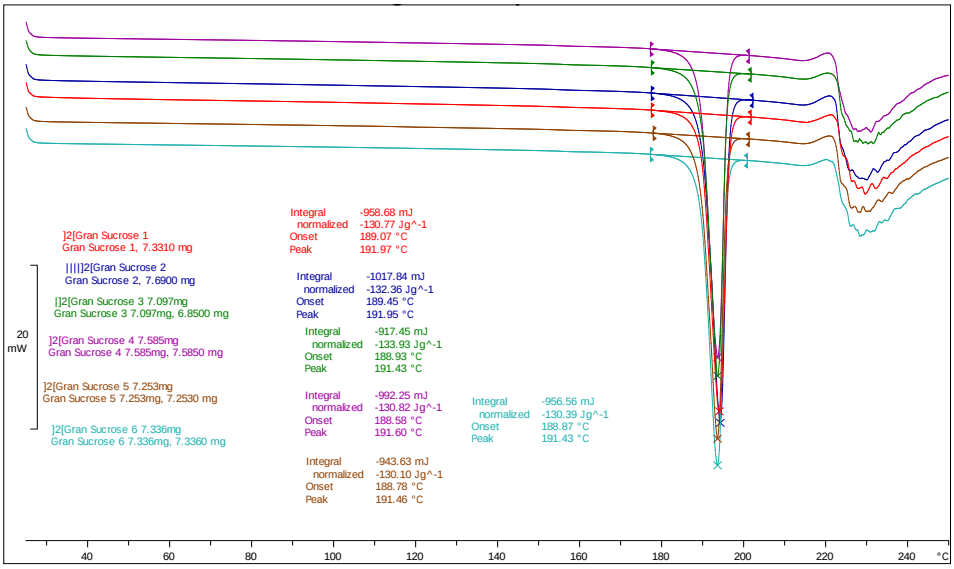

Figure 4.3 DSC Curve of crystalline Silverspoon sucrose (6 replicates) (Carrera, 2010) where there is no sign of any endothermic peak at 153°C (preceding the melting point of sucrose).

To investigate the prospect of the formation of impurities causing the differences observed between the thermograms of the two types of sucrose, ion chromatography of the mixed standard solutions of sucrose, glucose and fructose was conducted (Figure 4.4). The obtained results were compared with those extracted from chromatograms attained for sucrose solutions (Figures 4.5 & 4.6). The chromatographic analysis demonstrated that Fisher sucrose and Silverspoon sugar exhibited purity levels of 99.7 % and 98.4% respectively.

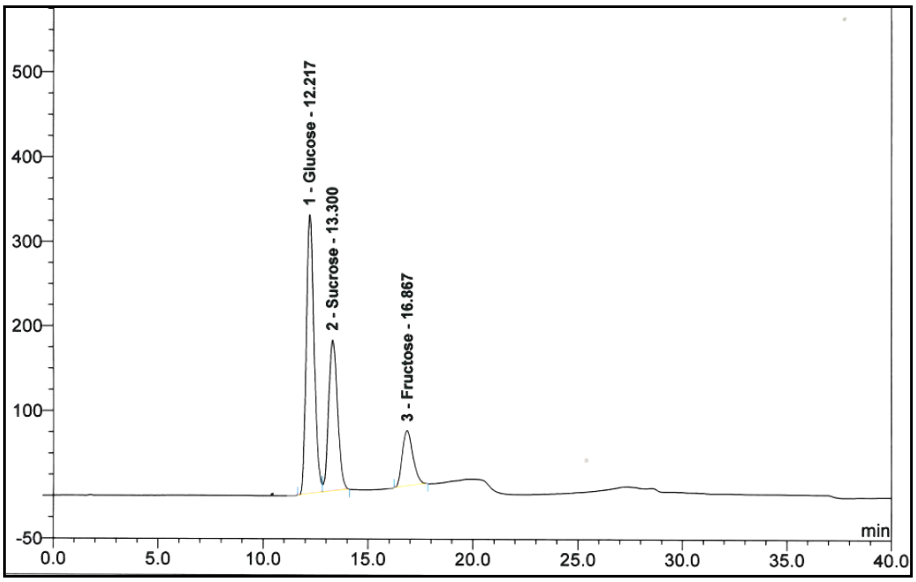

Figure 4.4 Ion chromatogram of mixed standards (sucrose, glucose & fructose)Carrera, 2010

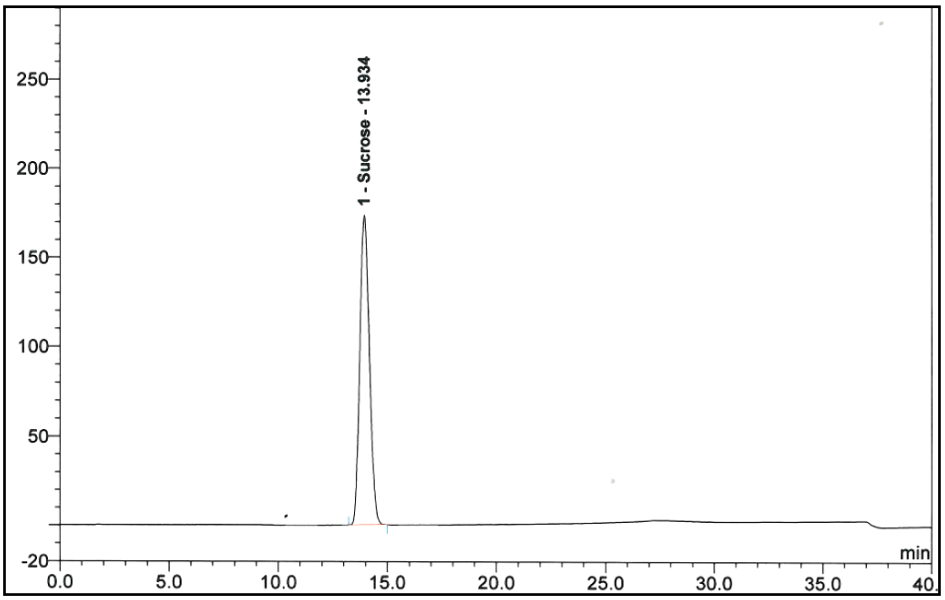

Figure 4.5 An ion chromatogram of Fisher sucrose – (Carrera, 2010).

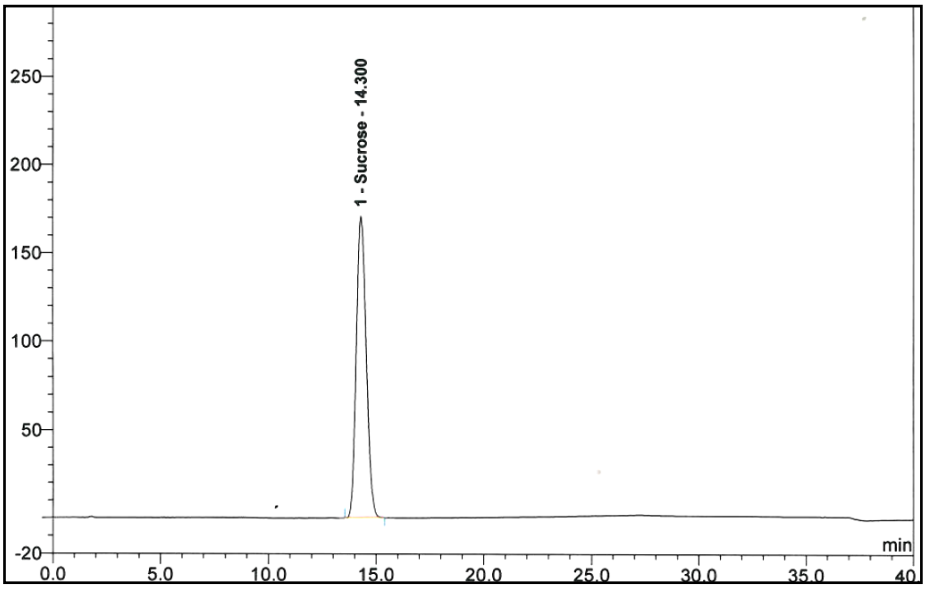

Figure 4.6 An ion chromatogram of Silverspoon sucrose (Carrera, 2010).

There are several sources of impurities in sucrose. Impurities could be monosaccharides (invert sugars), oligosaccharides (present in raw material such as raffinose in beet or kestoses and theanderose in cane), polysaccharides (e.g. dextrans), and inorganic non-sugars (e,g. potassium chloride) (Mathlouthi *et al.*, 1995). Minerals can be present in sucrose either due to the inorganic ash (mainly calcium and potassium salts) present in raw sugar or from occluded salts from the

crystallisation media used in the sucrose's production. Therefore, the samples were run using the multi-element screen method detecting about 30 elements on an inductively coupled plasma mass spectrometry ICP-MS (Perkin Elmer DRC II, Software version 3.0) equipped with an autosampler.

The most common inorganic non-sugars present in sucrose are sodium chloride and potassium chloride (Mathlouthi *et al.*, 1995). Based on the results obtained in this study, Silverspoon sucrose was detected with 5.50 ppm of potassium (K) and 1.40 ppm of sodium (Na) while Fisher sucrose was detected with no more than 0.01 ppm K and 1.20 ppm Na. Thus, Silverspoon sucrose contained a higher content of K and Na ions compared to Fisher sucrose.

4.3.2 Hot stage microscopy

Further investigations using hot stage microscopy were performed to visualize the thermal transitions taking place particularly during the lower temperature transition. Both Silverspoon and Fisher sucrose batches were monitored under the hot stage microscope where no phase transition was observed between 150 °C – 180 °C (Figures 4.7 & 4.8). The sucrose particles were liquefied at 204 °C, which is almost 10 °C beyond the melting temperature of sucrose peak in the DSC. Further investigations were carried out to investigate the potential sources of the melting point delay between the DSC and hot stage microscopy. A Silverspoon sugar sample was crushed into very fine particles and was analysed on hot stage microscopy. The melting point obtained for this sample was 200 °C which meant that reducing the particle size of the sample helped probably in increasing the thermal contact and thus speeded up the melting event.

Kuhnert-Brandstatter (1971) presented an identification table for the hot stage microscopy, where lidocaine (melting point = 68 °C) was reported to exhibit a melting at 72 - 73 °C. Moreover, Moraes *et al.* (2007) reported the melting point of lidocaine to be 77 °C measured by DSC at a heating rate of 10 °C/min. Consequently, lidocaine was analysed by hot stage

microscopy at three different heating rates 0.5 °C/min, 2 °C/min and 10 °C/min where the melting point for the first two heating rates was 73 °C while for the last one was 74 °C. Thus, the melting point of lidocaine determined by hot stage microscopy agrees with the range reported in literature. Therefore, the delay in melting point of the sugar samples between DSC and hot stage microscopy was considered acceptable.

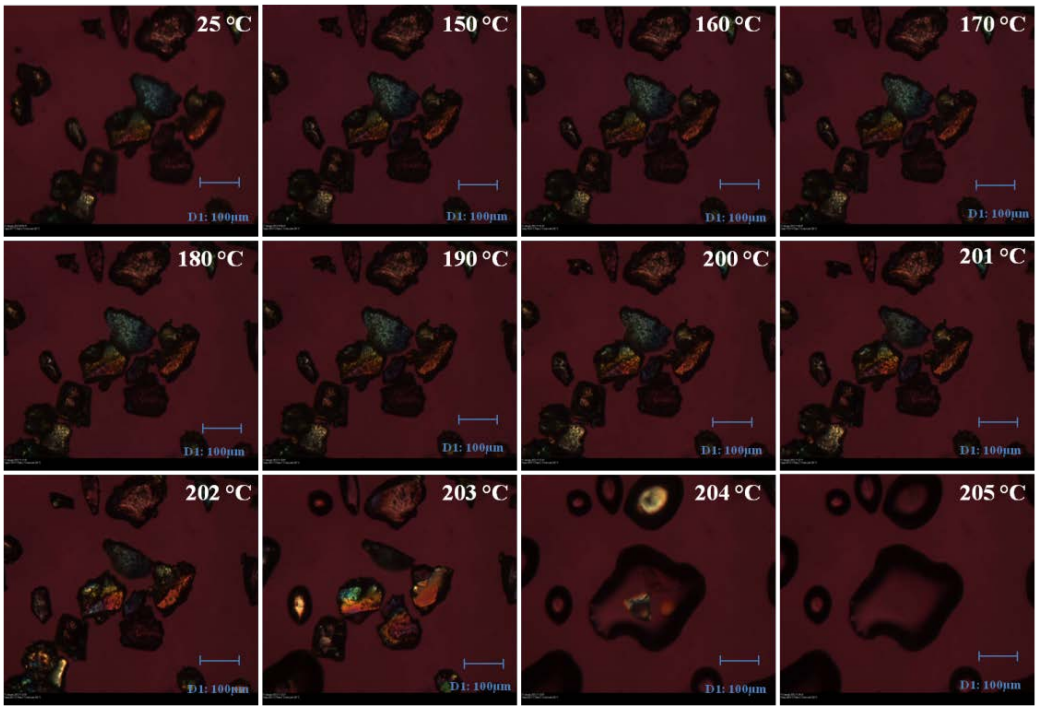

Figure 4.7 Hot stage microscope images of crystalline Silverspoon sugar, where no phase transition was observed between 150 °C – 180 °C (images generated from a slightly damaged polariser, remeded in chapter 5).

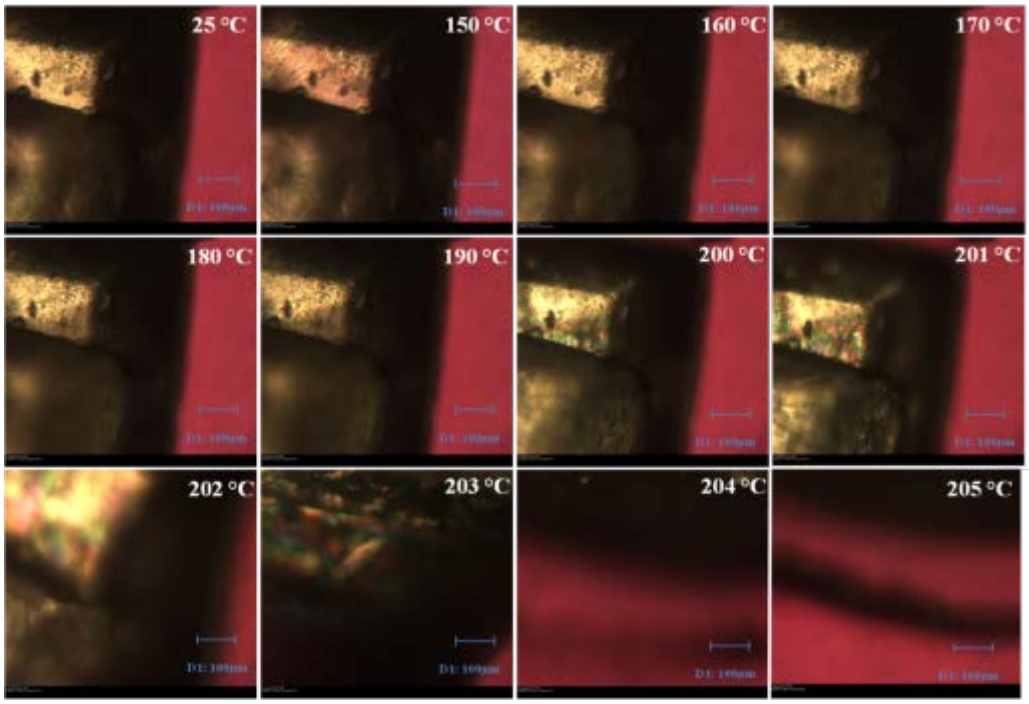

Figure 4.8 Hot stage microscope images of crystalline Fisher sucrose, where no phase transition was observed between 150 °C – 180 °C (images generated from a slightly damaged polariser, remeded in chapter 5)..

It was decided to use Silverspoon sucrose for the production of amorphous sucrose standards and its application in the model system. This is because Silverspoon sucrose is the sugar of choice in the chocolate crumb manufacturing process. Thus, it is preferable to use the same type of sugar in the model system to avoid any unanticipated differences in the minerals/ions profile between sugars.

4.3.3 Spray-drying of sucrose

Several attempts (with different experimental parameters) were performed to produce amorphous sucrose by spray-drying but proved ultimately futile. During the spray-drying of sucrose, water droplets started forming on the walls of the cyclone where the produced powder was collected. The experiment was halted; the product was checked and found to be a sticky syrup-like solution indicating the absence of any amorphous spray dried sucrose. The spray-drying of sucrose was repeated few times using the Buchi instrument without any ambivalent

success to produce amorphous sucrose powder. An alternative approach was considered where a Niro spray-dryer was used. The rationale behind using the "Niro" spray-dryer lies on its greater capacity compared to the Buchi analogue which prolongs the exposure time for the particles in the feed solution to hot air and therefore minimises the water content and potentially renders the final product less sticky. It has to be conceded that despite the numerous attempts made to optimise the procedure, producing a relatively dry sucrose powder using the Niro instrument was proven unachievable. Therefore, the production of amorphous sucrose by spray-drying was deemed inappropriate for this particular study.

4.3.4 Freeze-drying of sucrose

Freeze-drying produced a fluffy white powder cake in 7 mL glass jars (Figure 4.9).

Figure 4.9 A freeze-dried sucrose vial showing the dried amorphous sucrose cake.

4.3.5 Determination of water content by TGA

The water content of freeze-dried sucrose was determined by TGA and was found to be 1.2 % w/w ± 0.3 (n = 3, ± SD). The water loss (% w/w) for freeze-dried sucrose samples was determined by calculating the difference between the weight of the sucrose sample at 25 °C and that at 120 °C where the baseline was stable, indicating no more water loss (Figure 4.10).

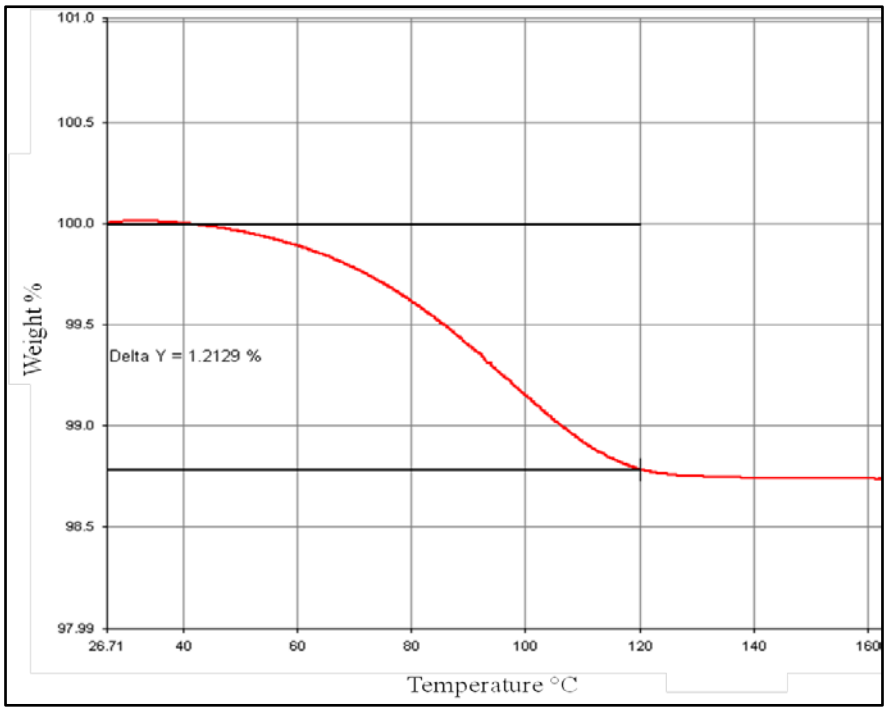

Figure 4.10 A TGA plot showing the water content of freeze-dried Silverspoon sucrose of 1.2% w/w.

4.3.6 Determination of optical rotation by polarimetry

The polarimer was calibrated using SigmaUltra sucrose. The observed optical rotation (OR) of three replicates of SigmaUltra sucrose was measured and found to be 63.5° ± 0.6 (SD, n = 3). For comparison purposes, three replicates of Fisher sucrose were also measured and the mean specific rotation was found to be 63.5° ± 0.5 (SD, n = 3). Three replicates of 10% w/v Silverspoon sucrose solutions were also measured and the mean specific rotation was estimated to be 65° ± 1.1 (SD, n = 3). It was noted that Fisher sucrose exhibited a similar specific rotation value to SigmaUltra sucrose, whereas, Silverspoon sucrose exhibited a slightly higher value. As mentioned earlier, it was decided that Silverspoon sucrose will be selected as a standard for the preparation and production of amorphous sucrose.

The optical rotation (OR) of all prepared solutions was measured (Table 4.2). The specific rotation $[\alpha_{Obs}]_D$ was normalised for solution concentration and pathlength in this study and it was calculated by applying equation 2 to the measured or observed optical rotation data.

Equation 2

Where = observed specific rotation, D = sodium D line monochromatic radiation (λ = 589 nm), α = observed optical rotation, l = pathlength in dm, C = concentration in g/100 mL (Shugar et a., 1996).

Table 4.2 The optical rotation of solutions of Silverspoon crystalline sucrose and its inverted forms.

Sucrose % w/w	Invert sugar % w/w (Glucose +Fructose)	Observed OR °		SD (n = 3)
100	0	6.5	65.4	0.3
75	25	4.9	49.8	0.3
50	50	3.2	31.9	0.1
25	75	1.1	11.4	0.4
0	100	-0.5	-5.2	1.1

The relationship between the specific rotation $[\alpha_{Obs}]_D$ and % w/w of the solutions used in the analysis was evaluated by constructing calibration plots. Linearity was assessed by fitting the results to linear regression analysis with the use of least square weighing factors ($1/x^2$). The calibration curve was linear over the range tested as demonstrated by the correlation coefficient (r^2 = 0.9981) (Figure 4.11). The freeze-dried sucrose sample was then measured on the polarimeter which produced an observed OR of 6.4° corresponding to a specific rotation $[\alpha]_D$ = 64°. This reading corresponds to a purity level of 98.5 % w/w and which asserts the absence of inversion upon freeze-drying. This 1.5% reduction (from 100% to 98.5%) may be due to the water content of the freeze-dried sucrose which was measured to be 1.2% w/w, thus reducing the concentration of sucrose in solution.

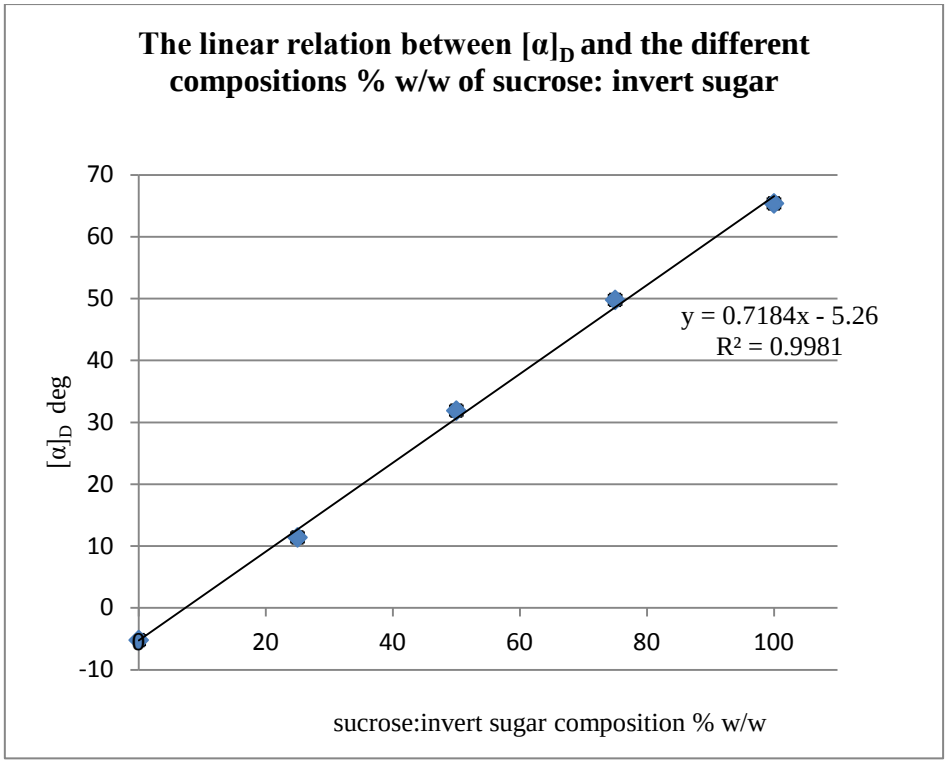

Figure 4.11 A calibration plot of different concentrations of sucrose: invert sugar (n = 3)

(Error bars based on SD are too small in range to be seen over the symbol used).

4.3.7 Characterisation of freeze-dried sucrose by DSC

DSC was used to characterise the freeze-dried sucrose. An overlay of three DSC replicates of amorphous freeze-dried sucrose illustrates that freeze-dried sucrose exhibits an initial step change corresponding to the T_g between 46 ºC and 50 ºC (Figure 4.12 & 4.13). This value agrees with the T_g range reported in literature (Roe, 2005). The glass transition is followed by loss of water from the sample due to the pin-hole pan. This is mainly due to a small amount of moisture (1.2 % w/w) embedded in the freeze-dried sample after finishing the secondary drying stage. A re-crystallisation exothermic peak is observed at ~ 110 - 120 ºC. Above the glass transition, the mobility of sucrose molecules increases dramatically and consequently the molecules are mobile enough to nucleate starting to gather up forming clusters which in turn form nuclei. Once the nucleation step has been overcome, nuclei grow into macroscopic crystals (Hartel, 1991). As the temperature rises, the sucrose sample starts to melt showing a melting peak at ~ 186-188 ºC. Further heating of the sample causes degradation (between 200 and 250

ºC) of sucrose which usually degrades at temperatures above its melting point (Mathlouthi *et al.*, 1995). The DSC thermogram of freeze-dried sucrose had confirmed the success of freeze-drying in producing a wholly amorphous sucrose sample.

Three replicates of the amorphous freeze-dried sucrose were overlaid to investigate the robustness and repeatability of the temperatures and heat capacities of both exotherms and endotherms. The three runs exhibit a relative standard deviation (% RSD) of 1.7, 2.2 and 0.4 % for the glass transition, re-crystallisation and melting point temperatures respectively. These results show high agreement among the different three runs which in turn reflect the repeatability of the DSC as a technique to confirm the amorphicity of sucrose.

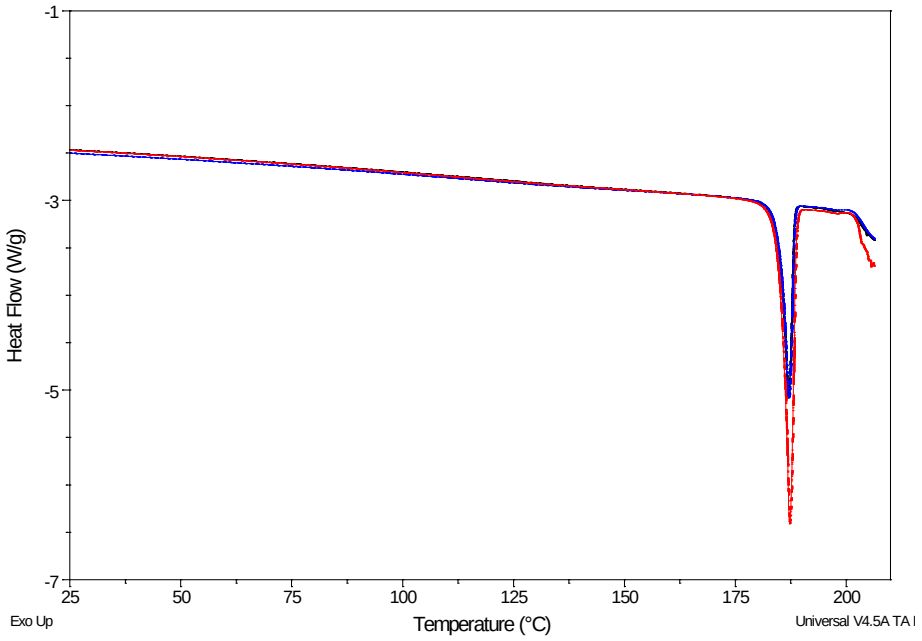

Figure 4.12 An overlay of DSC thermograms of Silverspoon crystalline sucrose (three replicates), showing the melting peak ~ 186-188 ºC.

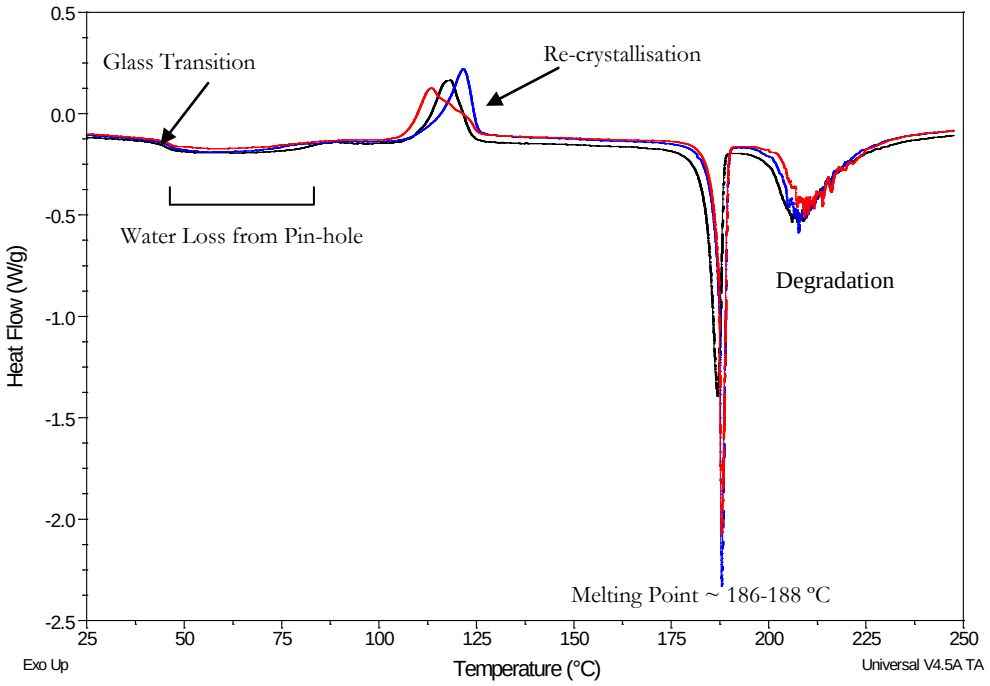

Figure 4.13 A DSC overlay of Silverspoon freeze-dried sucrose (three replicates), showing the glass transition, re-crystallisation, melting peak and degradation at higher temperature.

Table 4.3 A summary of the glass transition, re-crystallisation and melting peaks of Silverspoon freeze-dried sucrose (three replicates).

		Re-crystallisation Peak		Melting Peak	
	T_g (°C)	Temperature (°C)	Enthalpy (J/g)	Temperature (°C)	Enthalpy (J/g)
Rep 1	46.3	117.7	78.7	186.6	123.1
Rep 2	47.3	118.4	79.6	186.9	123.1
Rep 3	47.9	113.7	78.8	187.9	125.7
Mean	47.2	116.6	79.0	187.2	124.0
SD	0.8	2.5	0.5	0.7	1.5
RSD	1.7	2.2	0.6	0.4	1.2

Significantly, even though the shape of the re-crystallisation peak seems to be variable, the area of this peak is repeatable with an enthalpy of 79.0 J/g ± 0.95 (Table 4.3; Figure 4.13).

The glass transition was analysed by plotting two tangent lines across the step change corresponding to the glass transition with the point of intersection of both tangents representing

the glass transition temperature, as recommended by Haines (2002) (please refer to chapter 2 where an illustrave figure showing the analysis of the glass transition was included).

The agreement of the glass transition obtained with the values reported in literature (Roe, 2005) indicates that the freeze-dried sucrose produced has a high amorphous content. The mean enthalpy value obtained for re-crystallisation of the freeze-dried sucrose samples is 79 J/g (Table 4.3) which is very close to the enthalpy values of the re-crystallisation peak of freeze-dried sucrose reported in literature (71 J/g) (Gloria, 2001) and 65 J/g (Raemy, 1993).

Gloria and co-workers (2001) documented an approach for the quantitation of the amorphous content in freeze-dried sucrose samples. They used the enthalpy of the re-crystallisation peak to quantify the unknown proportion of amorphous content in the freeze-dried sucrose sample (equation 2). The approach was based on the assumption that the water activity (a_w) of amorphous sucrose was zero. However, no information or assumptions about inversion or degradation were considered.

$$\text{Amorphous sugar content (\%)} = \frac{\qquad\qquad}{\qquad\qquad} \qquad \text{Equation 2}$$

Where Enthalpy (J) is that for re-crystallisation of the freeze-dried sucrose under investigation and the enthalpy of re-crystallisation of a standard amorphous sucrose sample is assumed to be 71 J/g. When the results from the present study were placed into equation 2, the amount of amorphous content is 111%. This value indicates that the freeze-dried sucrose samples have a high amorphous content. The values obtained are higher than 100 % which might be either due to different water content of both freeze-dried sucrose samples or a difference in the integration parameters as neither the water content of the freeze-dried sucrose standards nor the integration parameters were reported by Gloria *et al.* (2001).

PXRD has been used in numerous laboratories to characterise freeze-dried sucrose samples which usually show a broad peak rather than sharp diffraction peaks observed in crystalline sucrose batches (Gloria *et al.*, 2001; Chinachoti *et al.*, 1986; Palmer *et al.*, 1956). Based

on the study conducted by Gloria *et al.* (2001) the freeze-dried sucrose with an amorphous content was identified by PXRD. However, diffraction patterns in PXRD were influenced by other variables such as the size and shape of the sugar crystals and the proportion of amorphous/crystalline sucrose (Gloria, 2001).

To conclude, the freeze-dried sucrose has been confirmed to be amorphous with a very high amorphous content when examined by DSC.

4.3.8 Characterisation of freeze-dried sucrose by DMA

DMA was utilised to characterise the glass transition and measure where the glass transition regions falls with respect to temperature.

In the DMA thermogram (Figures 4.14 & 4.15), the T_g onset at 10 Hz was determined as 43 °C ± 1.5 (± SD, n= 3). The T_g was observed by a modulus loss which could be attributed to the increase of the molecular mobility of the sample upon heating to temperatures higher than the glass transition of sucrose. This is followed by a re-crystallisation process which involves reconfiguration of the unstructured, randomly arranged molecules of the super-cooled liquid into a highly organized, three-dimensional, crystalline lattice with long range order. The resulting crystalline lattice will exhibit a strong modulus peak (at ~ 70 - 100 ºC) due to its strong elastic properties. The re-crystallisation process is usually followed by the melting peak which is observed through a sharp drop in the storage as the sample changes fully to a liquid. Therefore, a DMA thermogram of freeze-dried sucrose clearly confirmed a high degree of amorphicity of the material.

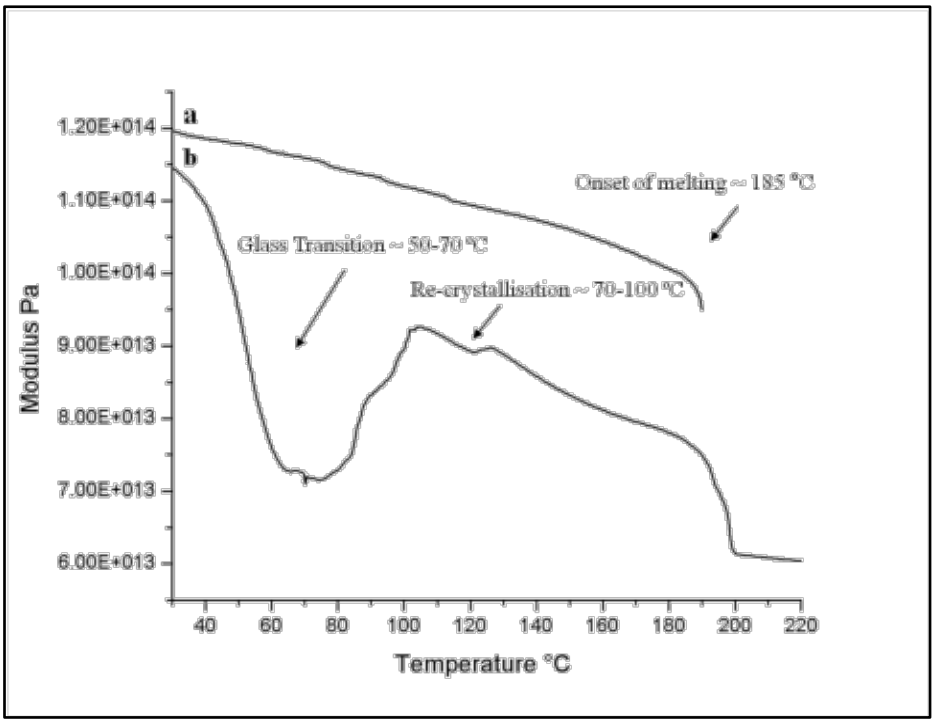

Figure 4.14 An overlay of DMA thermograms of Silverspoon sucrose (a) crystalline sucrose and (b) freeze-dried sucrose.

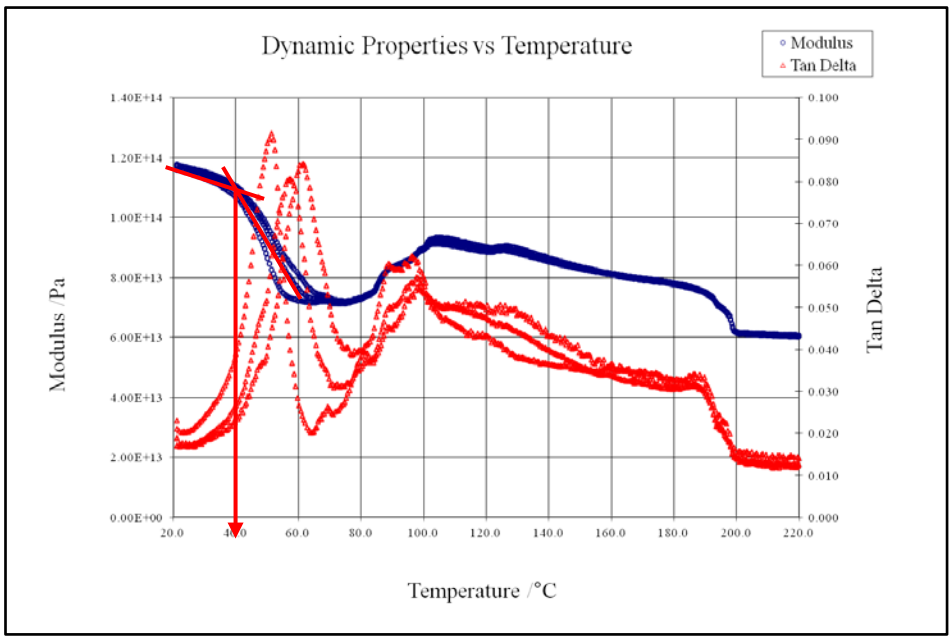

Figure 4.15 A DMA thermogram of Silverspoon freeze-dried sucrose (measurements taken at 3 different frequencies showing the frequency dependency of the glass transition).

4.3.9 Physical stability tests of freeze-dried sucrose

The aim of this investigation was to ensure that the produced amorphous sucrose was not re-crystallising over time and that the optimum storage conditions were applied. As reported in literature, the re-crystallisation of amorphous sugar with time is a critical issue for storing amorphous materials (Haque, 2005).

Amorphous sucrose samples were stored in sealed jars in a dessicator over phosphorus pentoxide at 25 °C in temperature-controlled rooms. The samples were tested at initial time point t_0, 1 week, 4 weeks, 12 weeks and 24 weeks (Figure 4.16).

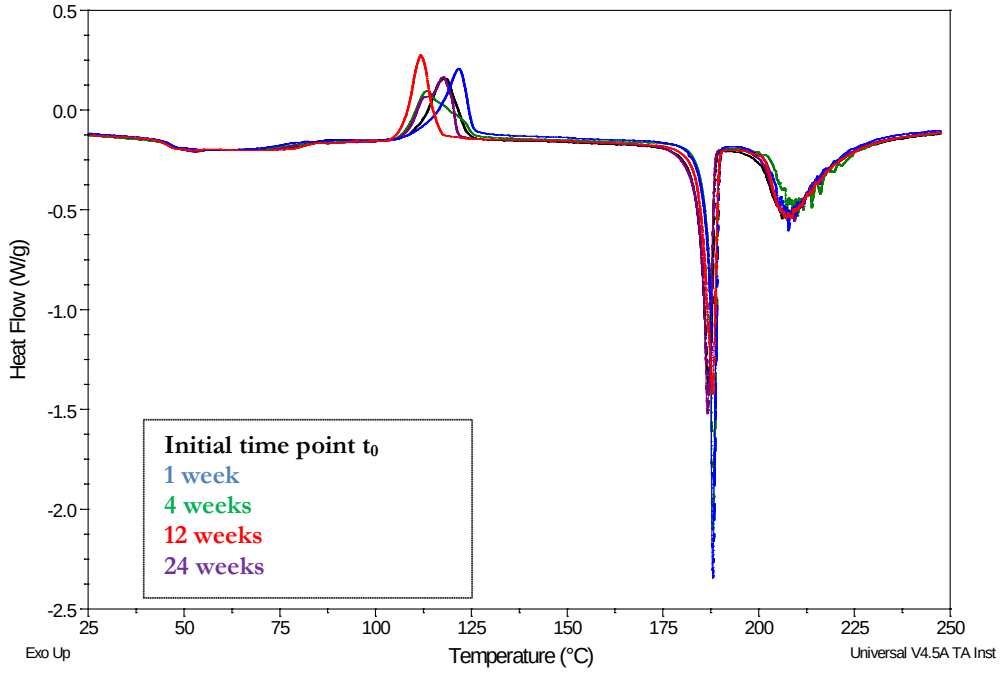

Figure 4.16 An overlay of DSC thermograms of freeze-dried sucrose samples at different storage time points.

Table 4.4 A summary of the glass transition, re-crystallisation and melting peaks of Silverspoon freeze-dried sucrose samples, five samples tested at 5 different time points.

		Re-crystallisation Peak	
	T_g (°C)	Temperature (°C)	Enthalpy (J/g)
Initial time point t_0	46.3	117.7	78.7
1 week	45.8	118.4	78.2
4 weeks	45.9	113.5	79.1
12 weeks	45.9	111.6	76.9
24 weeks	46.5	117.7	80.2
Mean	46.1	115.8	78.6
SD	0.3	3.0	1.2
RSD	0.6	2.6	1.5

The study demonstrated that sucrose remained amorphous over a period of 6 months (24 weeks) (Figure 4.16). The same pattern was observed for the DSC thermograms in all runs with similar glass transition, re-crystallisation and melting peak at very similar temperatures (Table 4.4). The T_g values of the five DSC runs were all similar ~ 46 °C ± 0.3. Furthermore, the areas of re-crystallisation peaks have not changed and were determined as ~ 78 J/g ± 1.6 which agrees with the crystallisation peaks of the initially prepared samples (79 J/g ± 0.5). If the initial material, as discussed previously, is assumed to be approximately 100 % amorphous; as the re-crystallisation enthalpy did not change over time, it is clear that upon storage, sucrose remained amorphous.

To conclude, the physical properties are not changing upon storage as the materials appear to be highly amorphous with no change or substantial crystallisation taking place upon storage.

4.4 Discussion

Sucrose is one of the main ingredients used in chocolate. Sucrose contributes to the rheological properties of the fat mixture which aids the flowability and solid formation behaviour of chocolate (Asadi, 2007). Moreover, amorphous sucrose may be introduced to chocolate during the different stages of the manufacturing process; thus, it is important to be able to

characterise the physical and chemical properties of amorphous sucrose and develop a clear understanding of the behaviour of these properties when amorphous sucrose is present in chocolate.

Therefore, the aim of this study was to establish a reproducible protocol for producing amorphous sucrose and determine any occurring hydrolysis upon freeze-drying. To achieve our aim, three main objectives were set. The first focused on determining the purity of crystalline sucrose prior to its use as a starting material for the production of amorphous sucrose. The second was to produce amorphous sucrose either by spray-drying or freeze-drying and lastly to determine the purity of the produced amorphous sucrose.

Prior to the use of crystalline sucrose in preparing feed solutions for freeze- and spray-drying experiments, it was tested for purity by DSC and IC. Both Fisher and Silverspoon sucrose batches were run on DSC in 3 triplicates and 6 replicates respectively. The DSC thermograms showed that Fisher sucrose exhibited an endothermic peak at 153.7 °C ± 0.4 (± SD, n = 3) while Silverspoon sucrose did not. Hot stage microscopy was applied as a qualitative confirmatory tool to support DSC and thus monitor the phase transitions that sucrose undergoes upon heating. Both sugars exhibited no phase transitions until the melting point. The complete melting of Fisher sucrose and Silverspoon sucrose occurred at ~ 204 °C, which is approximately 10 – 14 °C higher than the melting point obtained when the same the same crystalline sucrose samples was analysed on DSC (190 °C). A finely crushed Silverspoon sucrose sample was also monitored on hot stage microscopy to investigate whether the particle size of the sample was delaying the melting. The finely crushed sample had a melting point of 200 °C which was 4 degrees lower than the normal sample. This raised the hypothesis that this delay in melting might be directly linked to the particle size of the sample or the thermal lag within the sample because of the larger sample and lower area of thermal contact than a DSC. Future work that will investigate the difference between HSM and DSC would be to use a slower heating rate in the HSM, reduced sample size and to use validation materials for hot stage microscopes.

Lidocaine was used to validate the hot stage microscopy method by checking whether any delays in the melting point would be detectable. Kuhnert-Brandstatter (1971) reported that the melting point of lidocaine was 72 - 73 °C on hot stage microscopy; whereas the mean melting point of lidocaine is ~ 68 °C (ranging from 66 °C to 69 °C). Moraes *et al.*(2007) determined the melting point by DSC to be 77 °C. This suggests an almost 10 °C range of the melting point for lidocaine reported in literature, that shows a slight variation between hot stage microscopy and DSC. The experiments conducted in this study on lidocaine by hot stage microscopy produced results which agreed with the findings documented by Kuhnert-Brandstatter (1971). Therefore, this difference in melting between DSC and hot stage microscopy is considered acceptable. However, it has to be noted that hot stage microscopy did not provide any information on the endothermic peak preceding the melting of the Fisher sucrose samples i.e. the extra melting peak at 150 °C was not observed.

Another important observation comparing the two thermograms of Fisher and Silverspoon sucrose in DSC was that the melting peak of Fisher sucrose was broader than that of the Silverspoon sugar which possessed a sharp melting peak. The measured enthalpy of the melting peak of Fisher sucrose was 120 J/g ± 1 (± SD, n = 3) which is lower than the levels for, the melting enthalpy of sucrose reported in literature (134 J/g) (Hurtta, 2004) which is very close to the enthalpy measured for the Silverspoon sample (130 J/g ± 1.5). If the peak at 153 °C is a melt then some of the unmelted sucrose may dissolve in this molted material before the true melting of Silverspoon sucrose at 189 °C. This would also explain why the Fisher sample's melting enthalpy is lower at 186 °C.

The melting peak of sucrose has been previously studied as documented by several strands of experimental evidence where a very small endothermic peak prior to the melting peak was detected (Bhandari, 2002). It was postulated that this endothermic peak was a direct result of the presence of some amorphous fraction or surface solubilisation of the crystals by residual moisture (Bhandari, 2002). Maulny *et al.* (2004) also observed two endothermic peaks (at ~ 150

°C and 190 °C) in the DSC thermogram of crystalline sucrose, which agreed with the conclusions drawn from the study reported by Bhandari and co-workers (2002) regarding the endothermic peak at 150 °C.

Hurtta *et al.* (2004) also studied the melting behaviour of two sucrose samples: bulk and fine chemical. They reported that the bulk sucrose exhibited two endothermic peaks while one endothermic peak only was observed in the fine chemical sucrose. The occurrence of two endothermic melting peaks for the analytical grade sucrose has been reported by numerous independent investigators (Richards *et al.*, 1978; Eggleston *et al.*, 1996; Beckett *et al.*, 2006; Lee *et al.*, 2007; Lee *et al.*, 2011). The observed difference in the number of peaks between the analytical grade and the commercial batch of sucrose may be attributed to the presence of trace amounts of water or impurities e.g. salts, invert sugars (glucose and fructose) or organic acids which cannot be completely removed during the manufacturing process. The type and level of impurities, which vary among prepared sucrose samples by different manufacturing methods, may influence not only the number of endothermic peaks present, but also the melting temperature onset and the peak shape (Table 4.5) (Shah *et al.*, 1936; Hirschmuller *et al.*, 1953; Kamoda *et al.*, 1960; Beckett *et al.*, 2006). It is worth noting here that the presence of such impurities can accelerate sucrose decomposition (Kelly *et al.*, 1978; Kelly *et al.*, 1979; Richards *et al.*, 1986; Eggleston *et al.*, 1996; Clarke *et al.*, 1997).

Therefore, it was hypothesised that this extra endothermic peak and the reduction of the melting peak enthalpy might be a consequence of the presence of invert sugars in the form of glucose/fructose or the existence of small amount of mineral salts introduced during sugar processing.

Table 4.5 Literature values of melting temperatures of D-sucrose, D-glucose and D-fructose (Hurtta *et al.*, 2004).

Reference	Melting temperature (°C)		
	D-Sucrose	**D-Glucose**	**D-Fructose**
Shallenberger and Birch, 1975	160-186	146 (α)	102-104
		148-150 (β)	
Broido *et al.*, 1966		146	
Roos, 1993	(173) 190	(143) 158	(108) 127
Raemy and Schweizer, 1983	(160) 185	(135) 150	(80) 115
Slade and Levine, 1988	192	158	124
Ramos-Sanchez *et al.*, 1988	180	156	121
Fan and Angell, 1995			105
Saleki-Gerhardt and Zografi, 1994	188		
Orsi, 1973		165	120
Gloria and Sievert, 2001	188		
Vanhal and Blond, 1999	190		
Lide, 1994	185	146 (α)	103-105
		148-150 (β)	

*the values in parentheses are onset temperatures

To investigate the presence of invert sugars in sucrose, the endothermic peak temperature at 153 °C was compared to literature values to assess whether it corresponds to glucose or fructose. It was found that this endothermic peak lies within the literature value melting point range of glucose. The next step was to screen for the presence of invert sugars in the Fisher sample while Silverspoon sugar was used as a reference standard to compare both samples. Ion chromatography was selected as the method of choice to check for the presence of invert sugars.

Comparing the ion chromatograms of sucrose, fructose and glucose obtained from ion chromatography showed that there was no invert sugar present. These findings agree with the accumulated evidence reported in literature. Maulny *et al.* (2004) who studied co-crystallisation of mixtures of sucrose, fructose and glucose recorded that the tiny endothermic peak at ~ 150 °C that was present in these co-crystals persisted when sucrose was analysed in the absence of the invert sugars. This suggests that it is not linked to the presence of fructose or glucose in the crystalline form of sucrose. They also reported that upon increasing the level of added invert

sugar, no significant change in the enthalpy was observed for the first peak. Beckett also introduced 1 % w/w invert sugars in a sucrose sample exhibiting the endothermic peak at 150 °C and reported that there was no significant change observed (Beckett *et al.*, 2006). It was suggested that the presence of the endothermic peak at ~ 150°C in the DSC thermogram of sucrose is linked to its mineral salt content as adding mineral salts (Na and K) to the Fisher sucrose solution resulted in a complete removal of the peak at ~ 150 °C (Beckett *et al.*, 2006). This extra endothermic peak at ~ 150 °C might be due to the presence of a cocrystal (crystalline structure made up of two or more components in a definite stoichiometric ratio), where the type of cocrystal can be either a hydrate (containing water) or a clathrate (a lattice of one molecule trapping another one), in this case, mixtures of sucrose and very small amounts of mineral ions.

To determine the mineral content of the sugar samples, both Fisher and Silverspoon sucrose were analysed by ICP-MS (inductively coupled plasma mass spectrometry) to check for any differences in the mineral salts content between Silverspoon and Fisher sucrose. The basic principle behind ICP-MS is based on coupling an ICP which produces ions with a mass spectrometer to separate and detect the generated ions. The obtained results illustrated that Silverspoon sucrose was detected with 5.50 ppm of K and 1.40 ppm of Na while Fisher sucrose was detected with less than 0.01 ppm K and 1.20 ppm Na. Silverspoon sugar was found to have a higher mineral salt content than Fisher sucrose which inhibits the formation of endotherm peak at 153 °C as confirmed by the conclusions drawn from the study reported by Beckett which also suggested that the level of salts present can also be influenced by the processing units used when refining the sugar (2006). Hence, it was decided that Silverspoon sugar would be selected as a starting material to prepare the feed solutions prior to spray- or freeze-drying.

The selection decision can also be directly linked to the chocolate manufacturing process. The sugars that are usually used in chocolate manufacture are commercial sugars which contain higher mineral salts content. Therefore, they do not exhibit an extra endothermic peak prior to

the melting peak of sucrose. This is considered of critical importance when analysing DSC thermograms of any chocolate crumb samples.

Two techniques were investigated for the production of amorphous sucrose: spray- and freeze-drying. Several attempts were made to produce amorphous sucrose by spray-drying but they proved to be ultimately unsuccessful. This might be due to the outlet temperature of the spray-dryer which exceeded the glass transition of sucrose and prevented the formation of solid material. Conversely, when the outlet temperature was not sufficiently high, drying of the aerosols was incomplete leading to the formation of a syrup-like sticky material instead of an amorphous powder. Moreover, high outlet temperatures (above 180 ºC) might lead to thermal degradation of sucrose (Bhandari *et al.*, 1997). Hence, spray-drying was not the method of choice for the production of amorphous sucrose.

Freeze-drying of 10 % w/v aqueous sucrose feed solutions was performed according to the protocol reported in literature (van Scoik *et al.*, 1990; Kedward *et al.*, 1998; Chen *et al.*, 2001; kawakami *et al.*, 2006; Alkhamis, 2009). Freeze-drying was successfully applied to produce a relatively dry powder cake. The water content of the produced powder was determined by TGA and found to be 1.2 % w/w ± 0.3 (± SD, n = 3). The water content was deemed highly suitable for the investigations reported in this study as it resembles the final water content in the chocolate crumb (discussed in Chapter 5).

Prior to any thorough analysis concerning the amorphous content of the sample, it was important to ensure that the freeze-dried sucrose was chemically pure and hydrolysis/inversion-free upon freeze-drying. So, the chemical purity of freeze-dried sucrose was investigated. Polarimetry was the method of choice as it is very appropriate for detecting low levels within the amorphous material. Thus, the optical rotation was measured by polarimetry exploiting the chirality which sucrose possesses. The specific rotation of different compositions of sucrose: invert sugar solutions was measured, and the degree of purity of freeze-dried sucrose samples was deduced from the regression analysis which was applied to evaluate the relationship between

the specific rotation and different compositions used. The values obtained were in perfect agreement with those reported in literature (Mathlouthi *et al.*, 1995). The specific rotation $[\alpha_{Obs}]_D$ of 100 % sucrose solution was + 66.3º and the specific rotation $[\alpha_{Obs}]_D$ of 100% hydrolysed sucrose was - 20.7º. Amorphous sucrose was found to be 98.5 % w/w pure. This 1.5 % impurity could be due to the presence of water molecules (water content of amorphous sucrose was determined to be 1.2 % w/w) in the freeze dried sucrose. Therefore, negligible inversion took place over the freeze-drying cycle. It is worth noting here that ion chromatography (IC) was not used to investigate the purity of the amorphous sucrose as the IC method required too much material thus IC was used to validate the purity of crystalline sucrose while polarimetry was applied to measure the purity of the amorphous sucrose material.

After confirming the chemical purity of freeze-dried sucrose, it was essential to characterise the produced samples and investigate their physical transitions. The samples were then analysed by two different thermal analytical techniques: DSC and DMA. The DSC thermograms showed a glass transition at 46 - 50 °C which agrees with the reference values reported in literature (Roe, 2005). As the sample was heated, the mobility of the molecules gradually increased thus initiating nucleation and crystal growth. Ultimately, re-crystallisation occurs followed by melting of the sample. The amorphous content of the sample was also quantified by following the calculations reported by Gloria *et al.* (2001). Therefore, DSC was able to characterise the freeze-dried sucrose with high amorphous content.

DMA was also used as a supporting technique to characterise freeze-dried sucrose where changes in the viscosity and modulus of the samples were interpreted in terms of glass transition, re-crystallisation and melting which give an indication of a freeze-dried sucrose sample with a high amorphous content. Both techniques (DSC and DMA) confirmed the presence of glass transition which agrees with literature values, followed by re-crystallisation peak and then melting of the crystals. These results further validate the characterisation of freeze-dried samples with high amorphous content.

The physical stability of amorphous sucrose was also investigated by DSC over a period of 24 weeks to assess the efficiency of the storage conditions. Amorphicity was maintained in all the samples throughout the testing period as demonstrated by the repeatability of the T_g, re-crystallisation and melting peak temperatures evaluated by DSC.

It can be concluded that stable amorphous sucrose was successfully produced with a high degree of purity by freeze-drying.

4.5 Conclusion

Silverspoon sugar was used as the starting material for all of the sucrose standards that are reported here and in the next chapter. This selection was mainly based on the fact that the sucrose used in chocolate manufacture is more similar to Silverspoon. A highly stable and optically pure amorphous sucrose was successfully produced by the freeze-drying technique.

Chapter five – The crystallisation of sucrose in the presence of lactose, minerals and residual water

The aim of this study is to evaluate the impact of three independent variables on the crystallisation of sucrose in a model system using differential scanning calorimetry (DSC). The first two variables are mineral and lactose content. The third parameter was the sealing environment within the sample pan, which either allows or prevents the loss of residual water from the model system. This study provides a better understanding of the nature of interactions among the different variables, and their influence on the crystallisation behaviour of sucrose from its amorphous state.

5.1 Introduction

Chocolate crumb is commonly manufactured as a base ingredient for the production of milk chocolate (Minifie, 1980). Chocolate crumb is key to the flavour of milk chocolate. The manufacturing process of the crumb involves mixing milk, sugar and cocoa mass at a high temperature to drive off the moisture under high vacuum (Minifie, 1980; Beckett 2006). The optimal moisture content of the final crumb falls within the range of 0.8 % w/w and 1.5 % w/w because at this level the water activity is very low which inhibits the growth of microorganisms (Beckett, 2006). Therefore, crumb can be defined as a vacuum dried crystalline mixture of sugar, milk and cocoa solids (Figure 5.1). During the manufacturing process, sucrose (sugar) is added to milk to produce sweetened condensed milk (approximately 75% w/w dry solids), followed by the addition of cocoa solids. The final drying stages of the procedure, including the temperature and the heating time interval of the sugar with milk, confer to the crumb its unique caramelisation flavours and structural quality (Lees *et al.*, 1973; Minifie, 1980). The caramelisation flavours are a product of the Maillard reaction which depends on time, temperature and the moisture content of the material. Hence, chocolate crumb may contain agglomerates of crystalline sugars (sucrose and lactose) and cocoa solids bound together by an amorphous phase of protein, fats and amorphous sugars (Lees *et al.*, 1973).

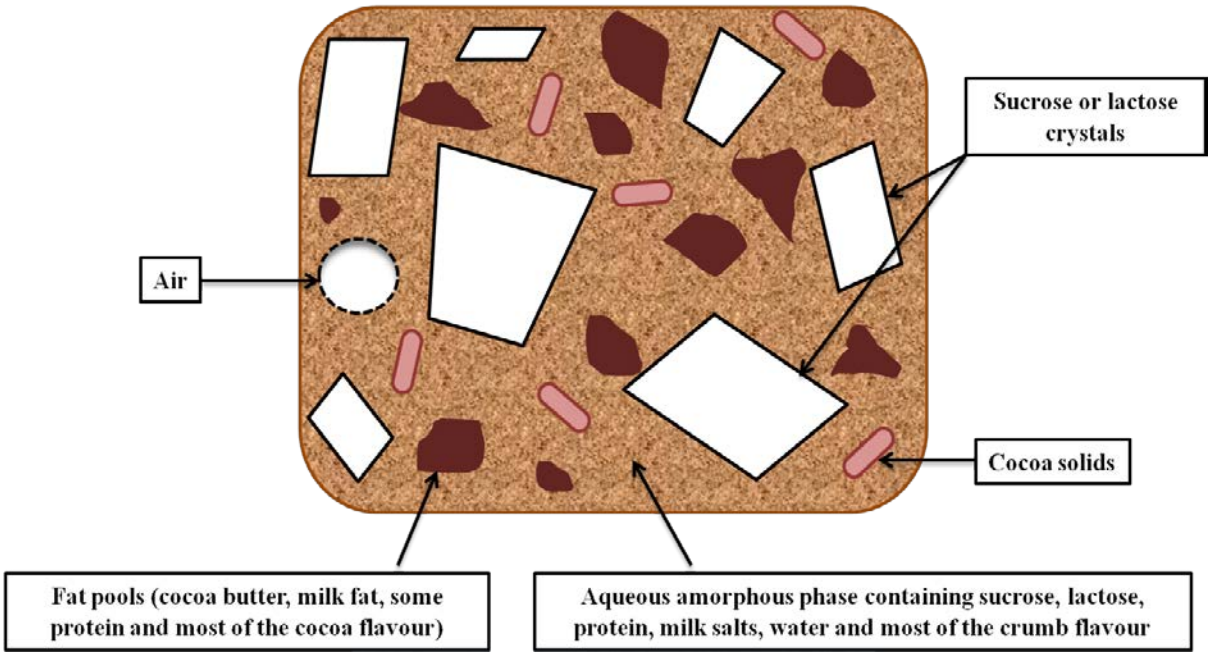

Figure 5.1 A diagram representing chocolate crumb structure with its various ingredients of sugars (sucrose and lactose), cocoa solids, cocoa butter, milk salts and protein.

The equipment in which chocolate crumb is prepared is typically a batch reactor operated under vacuum and in which heat is applied during the downstream drying stage (Figure 5.2). As the ingredients are processed, they are first mixed and then heated where elevated temperatures initiate the Maillard reaction, which creates significantly distinctive flavours (Minifie, 1980). The manufacturing process includes a pre-mixing step in which dry and liquid components, such as milk powder or condensed milk, sugar and cocoa liquor, enter the reactor. The mixture then undergoes a drying step that transfers the homogeneous mass changes from a pasty consistency to a dry, crumbly and free-flowing product.

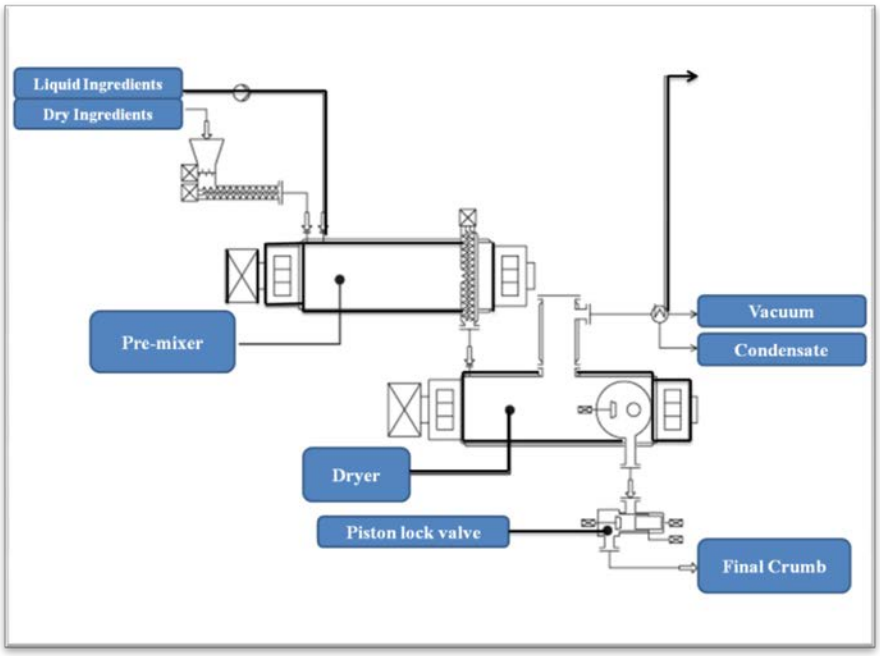

Figure 5.2 A diagram demonstrating a generic crumb manufacturing procedure (derived from www.list.ch).

It is important here to explain the link between the actual crumb manufacturing process and the study outlined in this chapter. As the crumb in a reactor is prepared under reduced pressure, water is drawn off continuously during cooking. Ideally, the study should track crystallisation of sucrose from a saturated solution rather than an amorphous phase. However, this is difficult to achieve in a DSC sample pan; crystallising from the melt is also an issue as sucrose might undergo thermal degradation upon melting, and thus a concern has been raised about the purity of the material (Lee *et al.*, 2011; chapter 4). Therefore, it was decided that the amorphous form of sucrose would be selected as the starting material. It had recently been demonstrated that re-crystallising sucrose from the melt phase in the DSC required that a sealed pan be used for containing the sample (Reading Scientific Services Limited RSSL, personal communication). Therefore, the presence or absence of residual water was chosen as one of the three variables to be investigated. The approach was based on applying hermetically sealed DSC pans versus pin-holed DSC pans. The pin-holed pans are more related to the reactor because

they are running under nitrogen (N_2) where any water vapour will be removed; thus, in pin-holed pans, the water vapour is replaced in the DSC by N_2 gas. Whereas, the hermetically sealed pans were used to investigate the crystallisation behaviour of sucrose in the presence of a small amount of trapped residual water mimicking an increase in pressure. Water is required for the crystallisation of sucrose in the reactor; thus, once water is removed by reducing the pressure, crystallisation ceases resulting in a proportion of amorphous sugars in the final crumb. Mathlouthi *et al.*, (1998) reported that controlling water vapour pressure during crystallisation was a key factor in determing which polymorph of a particular sugar was formed. Thus, moderating the crystallinity of the sugars in the crumb could occur by controlling the pressure during phase changes.

Minerals are a basic constituent of whey powder (Nijdam *et al.*, 2007). The more acidic the whey, the higher the mineral content is. This may generate off-flavours in the chocolate and accounts for the implementation of certain processes which favour the use of demineralised whey powders (Beckett, 2000). The mineral salts content of milk have been reported in literature (Rodriguez *et al.*, 2001; Fox *et al.*, 1998) (Table 1.4 in chapter 1). Milk and/or whey powders, containing minerals, are components of the crumb. It had already been demonstrated by RSSL that minerals played a significant role in impairing the crystallisation kinetics of sucrose during crumb processing (Elleman C.J., personal communication). The literature provided several possible reasons for this, minerals are known to sequester water due to the water-cation interactions (Elleman C.J., personal communication; Santagapita *et al.*, 2008; Chen *et al.*, 2005). Santagapita and co-workers (2008) reported that minerals have an impact on crystallisation temperatures and enthalpies of sugars. Sodium chloride (NaCl) was chosen as the test mineral because it is freely available, highly soluble and its chemical nature is close to that of potassium which is prevalent in whey powders.

The concept was based on investigating the impact of the mineral content at three different levels:

- Low: 0% w/w NaCl.
- Medium – 1.5% w/w NaCl
- High – 3% w/w NaCl

A mineral-free sample (0% w/w NaCl) was employed in the study in order to explore the impact of lactose on the re-crystallisation of sucrose in isolation (without the influence of any minerals). The mineral ratios chosen were 1.5% w/w and 3% w/w NaCl. The rationale behind selecting 3% w/w NaCl for high mineral content was based on typical levels of free minerals as a percentage found in certain crumb formulations (RSSL, personal communications). In all these formulations, the "total" mineral content as a percentage (% w/w) of sucrose is in fact higher due to "bound" minerals i.e. those associated with the casein micelle (Fox *et al.,* 1998). It has to be conceded that the above ratios are based on typical "total" mineral content as the differences between "free" and "bound" are yet to be fully understood. To date, no extensive work has been done on the DSC traces of "bound" systems.

The two major sugar components of chocolate crumb are sucrose and lactose. These two sugars can exist in either crystalline or amorphous forms. The final crystallinity of sugar agglomerates in the crumb varies depending on the thermal profile of the crystallisation process. Lactose can exist in its amorphous or crystalline forms including α-lactose monohydrate, anhydrous β-lactose or a combined ratio of both (as detailed in chapters 1 & 2). Crystallinity in crumb is thought to be of significance as the level of free fat is found to be proportional to the crystallinity of the sugars (Biagrie *et al.*, 1988). As crystallinity increases, the level of free fat increases accordingly.

The level of amorphous lactose present in the finished chocolate product may influence the mouth feel of the chocolate. This could be accounted for by the hygroscopicity of

amorphous sugars which possess a high tendency to absorb water. The subsequent increase in moisture content enhances the stickiness of the sucrose crystals building a skeleton of tightly bound sugars which increase the perceived cohesiveness of chocolate in the mouth (Beckett, 2006). Thus, a better understanding of the physical properties of the individual sugars (lactose and sucrose) in crumb and their impact on one another is an area of interest for this study.

Lactose possesses relatively low solubility (7 g/100mL at 25 °C) compared to sucrose which exhibits a solubility of 200 g/100 mL at 25 °C (Fox *et al.*, 1998; Mathlouthi *et al.*, 1995). The solubility of both sugars tends to increase as temperature rises. However, the concentration at which they reach supersaturation depends on other solutes present in solution. Therefore, the presence of either sugar in a solution may lower the crystallisation concentration of the other due to the preceding supersaturation (Table 5.1). Nickerson and colleagues (1972) have shown that increasing sucrose concentration can reduce lactose saturation (Table 5.1). Another independent investigation conducted by Livney and co-workers (1995) have illustrated that sucrose is capable of inhibiting the nucleation process of lactose.

Table 5.1 Solubilties of lactose in sucrose solutions, values generated by Nickerson *et al.*,1972.

Concentration of sucrose in water (% sucrose in total 100g)		40% w/v solution of sucrose in water	50% w/v solution of sucrose in water	60% w/v solution of sucrose in water	70% w/v solution of sucrose in water
Temperature °C	Solubility of lactose in pure water	Lactose: weight % which will dissolve in the solutions of sucrose			
25	19 %	14	12	10	8
40	27 %	20	17	14	12
50	32 %	24	21	17	14
60	38 %	31	27	22	21
80	49 %	44	38	35	32
85	52 %	42	38	35	33

Where the crumb making process goes through a drop in temperature during crystallisation, this might lead to the formation of amorphous sugars in the crumb (Beckett,

2006). Lactose has a documented glass transition (T_g) of 116 ºC (Roos, 1995) while sucrose exhibits a T_g ranging between 50 - 75 °C (Roe *et al.*, 2005). Because the two sugars differ in their T_g values, the crystallisation behaviour and the onset temperatures of re-crystallisation are different for both. Crystallisation only proceeds when there is sufficient energy and steric space for the molecular reorganisation during crystallisation to occur. Upon cooling, once the glass transition is reached then any amorphous phase becomes stable. Crystallisation may take place below this temperature threshold but it requires the presence of water which effectively lowers the T_g (Hancock *et al.*, 1994; Ibach *et al.*, 2007). As previously mentioned, lactose has a higher T_g value than sucrose which means that during cooling of the crumb, sucrose spends more time above its T_g than lactose does. Once the crumb manufacturing process is complete, the temperature of the crumb is unlikely to exceed the glass transition of sucrose while lactose is likely to be stable for further crystallisation to take place, higher temperature combined with higher water content is needed.

Brostow and colleagues (2008) reported the calculations of T_g values as a function of the composition where a fully miscible system is characterised by a single T_g. In compatible systems, two T_g values exist while in immiscible systems, T_g does not change with composition (Figure 5.3). From a theoretical standpoint, this phenomenon can be explained by the Gordon-Taylor equation which predicts the T_g of a binary component system based on the T_g, the densities, and the weight fractions of the pure components (Brostow *et al.*, 2008; Seo *et al.*, 2005; Levine, 2002; Lu *et al.*, 1992; Gordon *et al.*, 1952). The Gordon-Taylor equation is expressed as follows (Gordon *et al.*, 1952):

Equation 1

Where x_1 and x_2 are weight fractions or mole fractions of components 1 and 2 respectively, k is a constant (average of k values obtained by solving equation 1 for each component system).

Seo *et al.* (2005) have elucidated that the T_g of a disaccharide-disaccharide mixture can be computed by applying the Gordon-Taylor equation. They reported that a disaccharide-disaccharide component system obeyed the Gordon-Taylor equation without showing any deviation. Therefore, Gordon-Taylor equation can be applied to sucrose-lactose system to assess the final T_g of the mixture.

Brostow and colleagues (2008) also reported the calculations of T_g values as a function of the composition where a fully miscible system is characterised by a single T_g. In compatible system, two T_g values exist while in immiscible systems, T_g does not change with composition (Figure 5.3).

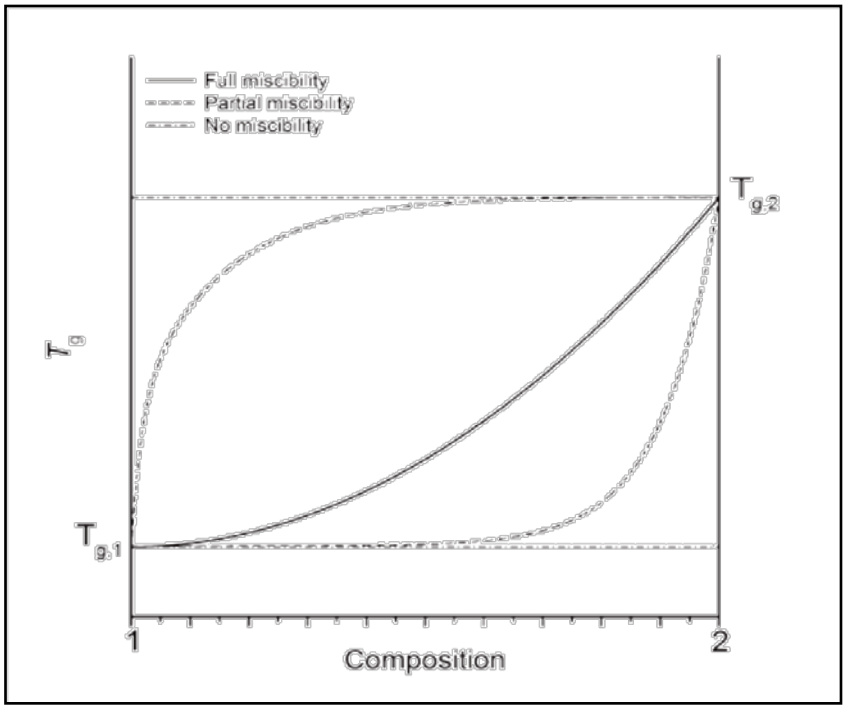

Figure 5.3 A schematic representation of the dependence of T_g on composition in binary blends (Brostow *et al.*, 2008).

It is noteworthy that further drying of the crumb may cause a rise in the T_g value due to the removal of water which if present lowers the T_g of both sucrose and lactose significantly. Roos *et al.* (1993) demonstrated that the presence of 5 % water can cause a fall of 40 °C in the T_g of lactose and 20 °C of the T_g for sucrose. The impact of water on the crystallisation process was

found to be the same on the temperature of crystallisation and T_g by a constant value of T_{cry} - T_g (Roos *et al.*, 1991a; Roos *et al.*, 1991b). Kedward and colleagues (2000) referred the shift in maximum crystallisation rate with water content to the changes in glass transition temperature T_g and melting point temperature T_m. As the crumb recipe is heated and water is removed, the mixture approaches supersaturation. At this point, some of the sugars will crystallise out which will cause a peak in the viscosity of the mixture. This is attributed to the dependence of viscosity upon both the physical form and temperature of mixtures. In other terms, if sucrose starts to crystallise (due to its supersaturation) then the viscosity of the whole mass will increase. This, in turn, will result in a lowering of the concentration of the other components which will only become supersaturated as more water is removed by the vacuum.

Kedward and colleagues (1998) reported that the reciprocal crystallisation half-time values of sucrose/lactose mixtures were lower than those reported for sucrose and lactose (Figure 5.4). This means that the rate of crystallisation is lower in mixed systems than in pure sugar. Kedward (1998) suggested that the rate determining factor of crystallisation in mixed systems could be the phase separation of the two components.

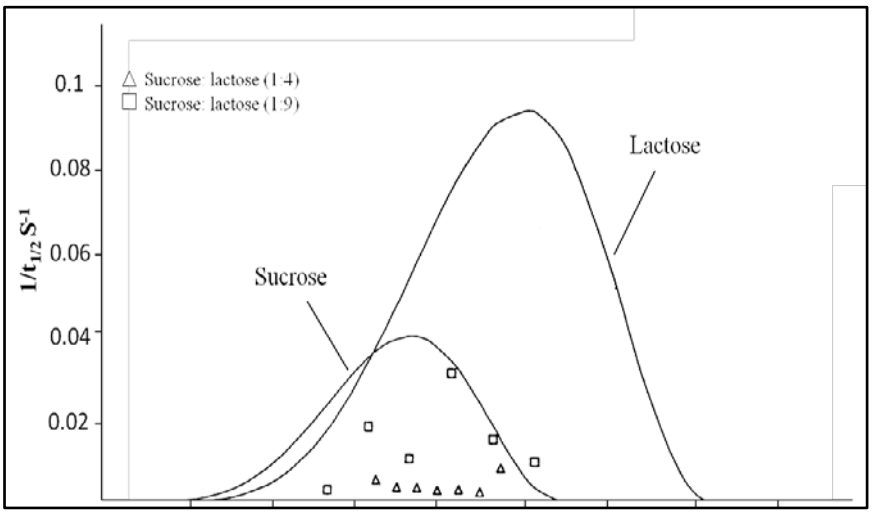

Figure 5.4 Reciprocal crystallisation half-time values of sucrose/lactose mixtures

(Kedward, 1998).

As discussed above, lactose can have a significant impact on the crystallisation of sucrose. Therefore, sucrose: lactose ratio was included in the variables under investigation in this study. Three main levels were chosen for sucrose: lactose ratios:

- Low – 0% lactose.
- Medium – 17% w/w lactose.
- High – 34% w/w lactose.

A lactose-free sample (0% lactose) was prepared to explore the impact of minerals on the re-crystallisation of sucrose on its own. The medium level of lactose (17% w/w) represents the typical lactose content in a standard crumb while the upper level of lactose content (34% w/w) can be sometimes conceivable for crumb (RSSL, personal coomunications).

Because there are a number of factors which influence crystallisation, an experimental strategy is warranted to tackle the concept systematically, which is a design of experiments (DoE). DoE gathers the maximum amount of information possible on the interactions between different factors within the product under investigation. DoE involves a full matrix containing all possible combinations of factors and levels. It can be defined as a systematic planned approach for determining cause and effect relationships (Cox *et al.*, 2000; Tamhane, 2009). DoE can be applied to any process with measurable inputs and outputs. Examples of applications of DoE include development of new products and new processes, optimisation of an existing manufacturing procedure, minimisation of product cost, etc. The DoE is an efficient method of maximizing information gained where variation is present**.** A typical DoE involves a series of structured tests designed to monitor the interactions of planned changes to the input variables (controlled factors) of a process or system in which the response of such interactions is then assessed (Figure 5.5).

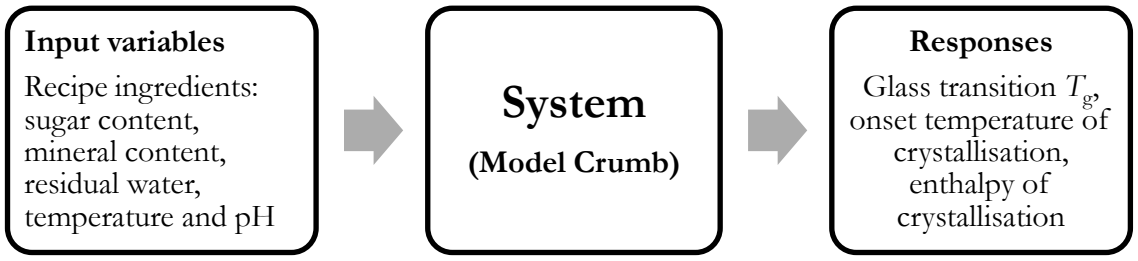

Figure 5.5 A diagram showing a DoE process applied to the model system.

DoE screens for the significance to the response of input variables acting alone, as well as the interactions of the input variables combined with one another i.e. whether input variables change the response on their own, when combined, or not at all (Atkinson *et al.*, 2007; Cox *et al.*, 2000). A typical DoE can test each level of one factor in combination with each level of all factors at the same number of times. So, DoE is a very time and money efficient method as, in terms of resource, it sets the exact length and size of the experiment. To set a DoE, two main parameters should be identified which are the input variables and the response (sometimes referred to as output) to be measured (Figure 5.5). Each input variable requires a number of levels to represent the range for which the effect of that variable is desired to be known. Figure 5.6 shows the essential stages of a typical DoE.

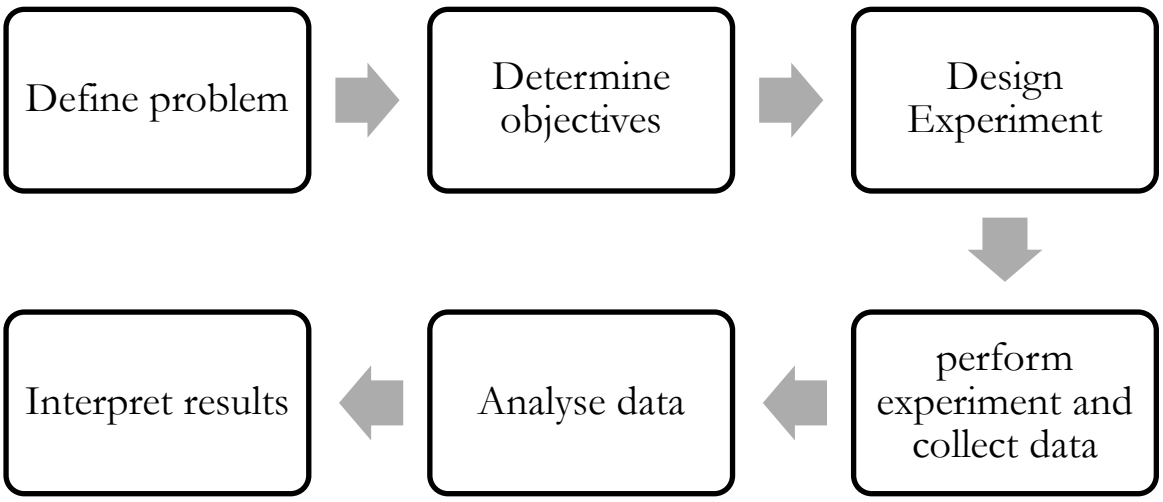

Figure 5.6 A schematic diagram that shows the essential stages of a typical DoE.

DoE experimentation relies on both replication (leading to substantial improvements in precision and allowing for an estimate of experimental error) and randomisation (mainly for avoidance of any bias) (Cox *et al.*, 2000). The benefits of experimental design include increased experimental efficiency, ensuring value-optimised products as well as competitive superiority. As the number of factors or number of levels of each factor increases, the number of required experiments becomes large. The number of experiments required can be calculated as follows: 3 factors with 2 levels $\Rightarrow N = 2^3 = 8$ experiments. The experimental work is usually followed by statistical analysis which targets collecting and analysing the appropriate data and drawing valid and objective conclusions. Responses are measured for all experiments and statistical analysis is followed to monitor any differences among responses belonging to different groups of input variables. These differences can be then attributed to the input variables acting alone (called a single effect) or in combination with another input variable (called an interaction). Statistical analysis normally involves analysis of variance and multiple linear regression of partial least squares.

In this project, three factors with respect to chocolate crumb were chosen in order to achieve the optimum conditions and to obtain a clearer understanding of interactions among the

different variables involved in the crumb manufacturing process. The three factors are mineral content, sucrose: lactose ratio and residual water content of the recipe. The selection criteria for choosing the three variables for the DoE have been discussed earlier in this section.

Manufacturing conditions for sucrose crystallisation during crumb manufacture were not achievable in the laboratory so it was decided to start with dry amorphous sugar/mineral mixes and run the study in the DSC with closed and pierced pans. The purpose of the pierced vs sealed pans was to give some regulation of vapour pressure during the thermal transitions. Therefore, the aim of the work reported in this chapter was to measure the crystallisation of sucrose in the presence of lactose and minerals. The research objectives to achieve this aim were to:

1. Establish a model system that contains sucrose, lactose and minerals which may be used to characterise crystallisation.
2. Apply the previously developed thermo-analytical method that monitors the thermal changes associated with crystallisation.
3. Develop a design of experiment methodology that will show how the constituents of the model affect the crystallisation of amorphous sucrose.

5.2 Materials and methods

The materials and instrumentations which have been used in this study and their details were discussed in chapters 2 & 4 in the materials sections are: sucrose, α-lactose monohydrate, phosphorus pentoxide, HPLC water, Gilson pipette pipetman, Aldrich atmosbag tape-seal, nitrogen gas cylinder, varian Girovac model GVD4 freeze dryer, DSC Q20 TA Instruments aluminium hermetic DSC pans and lids, NMR Brucker 400 Mhz, NMR tubes and DMSO, Hot stage microscope (DIALUX 22EB).

The materials that have not been reported in earlier chapters include sodium chloride NaCl (Fluka, Sigma Aldrich, UK), JMP® Statistical Discovery Software, version 10, supplied by SAS Institute Inc., USA and Karl-Fischer (Metrohm 870 KF Titrino).

5.2.1 Preparation of the feed solutions for freeze-drying

For freezing-drying, 10% w/v aqueous feed solutions of sucrose, sucrose with α-lactose monohydrate and sucrose with NaCl in different concentrations using crystalline material were prepared in HPLC grade water. The 10% w/v feed solution was with respect to all the solid material added. All solutions were prepared and stored at 25°C. In total, five 10% w/v aqueous solutions were prepared to give the following samples (Table 5.2):

- 100 % w/w sucrose with respect to the final freeze-dried sample (LL).
- 66 % w/w sucrose and 34 % w/w lactose solution (LH).
- 97% w/w sucrose and 3% w/w NaCl solution (HL).
- 81.5 % w/w sucrose, 17% w/w lactose solution and 1.5 %w/w NaCl (MM).
- 63 % w/w sucrose, 34% w/w lactose solution and 3% w/w NaCl (HH).

Table 5.2 Different sugars and NaCl compositions of the solutions before freeze-drying.

	LL	**LH**	**HL**	**MM**	**HH**
Wt. Sucrose (g)	5	3.3	4.85	4.075	3.15
Wt. Lactose (g)	-	1.7	-	0.85	1.7
Wt. NaCl (g)	-	-	0.15	0.075	0.15

5.2.2 Freeze-drying

The freeze-drying procedure was identical to the method detailed in chapter 4 section 4.2.3

5.2.3 Water content determination

The Karl-Fischer (Metrohm 870 KF Titrino) was standardised using the Karl-Fischer reagent hydranal standard (5- Standard sodium tartrate dihydrate) (Neuss *et al.*, 1951), where measurements were taken in triplicates. The samples were then analysed using hydranal

composite 5, and a solvent mixture of 1:1:1 methanol:formamide:chloroform.. Samples (0.2g) were analysed in duplicate. As the sample was titrated, the Karl Fisher oxidation reaction commenced producing iodine in proportion to the quantity of electricity so the water content was calculated based on the coulombs required for electrolytic oxidation.

5.2.4 Differential scanning calorimetry (DSC)

The DSC method used was described in detail in chapter 4, section 4.2.8.

5.2.5 Nuclear magnetic resonance (NMR)

The NMR method used was described in detail in chapter 2, section 2.2.8.

5.2.6 Hot stage microscopy

The thermal transitions of the amorphous sugars produced were observed directly by hot stage microscopy using a polarising microscope using the following method developed by Warren (2011). Hot stage microscopy was carried out using a Leitz Dialux 22EB microscope fitted with crossed polarisers and a λ plate (red 1 compensator) (Morris and Miles, 1994), with a Qi Imaging QiFastcam attached to a computer running the Linksys32 software package (Linkam). A minute amount of the freeze-dried sugar was smeared onto a coverslip (SLS laboratory supplies). Another coverslip was placed on top to sandwich the sample. The sample was then placed into a Linkam HFS91 heated stage, which was attached to the microscope, controlled by a Linkam TP92 controller. The TP92 control box was attached to the computer, so that the camera and heated stage could both be controlled simultaneously via the Linksys software package. The sample was then heated from 25 °C to 200 °C at 2 °C/min with a picture being taken by the camera every 10 s.

5.2.7 Design of experiments (DoE)

The DoE was set using the JMP software. A JMP data table is organized like a spreadsheet where columns correspond to variables and rows correspond to observations. The

data can be rearranged in the JMP data table with tables, rows, and columns menus. The variable input has been entered with their different three levels and the following DoE was generated (Table 5.3). The randomisation of the week, day, and order was used to eliminate any potential bias. Randomisation helps in distinguishing a 'true' experiment from a 'quasi' experiment and in turn enhances the precision of experiments.

Table 5.3 A table showing the randomisation of the week, day, and order of the three variables and their levels.

Week	Day	Order	Mineral	Ratio	Sealing	Symbol
1	1	1	High	Low	Sealed	HLS
1	1	2	Medium	Medium	Unsealed	MMU
1	1	3	Low	High	Sealed	LHS
1	1	4	Medium	Medium	Sealed	MMS
1	1	5	Low	Low	Unsealed	LLU
1	1	6	High	High	Unsealed	HHU
1	2	1	Low	High	Unsealed	LHU
1	2	2	High	Low	Unsealed	HLU
1	2	3	Medium	Medium	Sealed	MMS
1	2	4	High	High	Sealed	HHS
1	2	5	Low	Low	Sealed	LLS
1	2	6	Medium	Medium	Unsealed	MMU
1	3	1	Medium	Medium	Sealed	MMS
1	3	2	High	Low	Unsealed	HLU
1	3	3	Low	High	Unsealed	LHU
1	3	4	Low	Low	Sealed	LLS
1	3	5	Medium	Medium	Unsealed	MMU
1	3	6	High	High	Sealed	HHS
2	1	1	Low	High	Sealed	LHS
2	1	2	Medium	Medium	Unsealed	MMU
2	1	3	High	High	Unsealed	HHU
2	1	4	High	Low	Sealed	HLS
2	1	5	Low	Low	Unsealed	LLU
2	1	6	Medium	Medium	Sealed	MMS
2	2	1	Low	Low	Unsealed	LLU
2	2	2	Medium	Medium	Sealed	MMS
2	2	3	High	Low	Sealed	HLS
2	2	4	Medium	Medium	Unsealed	MMU
2	2	5	High	High	Unsealed	HHU
2	2	6	Low	High	Sealed	LHS
2	3	1	Medium	Medium	Unsealed	MMU
2	3	2	Medium	Medium	Sealed	MMS
2	3	3	High	Low	Unsealed	HLU
2	3	4	Low	High	Unsealed	LHU
2	3	5	Low	Low	Sealed	LLS
2	3	6	High	High	Sealed	HHS

5.4 Results

A measure of the crystallisation of sucrose from its amorphous freeze-dried form, in the presence of lactose and minerals when heated was used in this study to give an understanding of the fundamentals of sucrose crystallistaion in relation to the presence of minerals, lactose and at two different vapour pressures. The DoE employed in this study was conducted to evaluate the impact of different mineral levels (high vs. low), lactose: sucrose ratio (high vs. low) and sealing vs. unsealing on the enthalpy of crystallisation, the onset temperature of this re-crystallisation and the glass transition measured by DSC. The model was initially designed to test the mentioned interactions; however, the analysis produced required modifying as not all the responses generated could be used.

The water contents of freeze-dried sucrose and freeze-dried sucrose containing 3 % w/w NaCl were determined by Karl-Fischer analysis and were found to be 1.6 % ± 0.07 w/w and 2.3 % ± 0.1 (n = 2) respectively. This indicates that the freeze-dried sucrose containing 3% NaCl showed a higher water content than the samples without NaCl.

It was also important to confirm the purity of freeze-dried lactose in a mixed sugar system. Patel *et al.* (1970) reported that the mutarotation velocity of lactose slightly decreased by adding up to 40 % sucrose and decreased drastically thereafter. They also reported that the addition of some sodium and potassium salts catalysed and speeded up the mutarotation rate of lactose. Bekkum and co-workers (1996) illustrated that salts in whey do increase the mutarotation rate of lactose while sucrose decreases it. Nevertheless, it has to be conceded that the accumulated evidence reported in literature focused on the velocity of mutarotation rather than the final equilibrium ratio of the α- and β- anomers.

The NMR results from previous studies (chapter 2) showed that freeze-dried lactose in a single sugar system (with a standing time of 4 h of the feed solution), exhibited a β/α ratio of 60/40. Thus, all sucrose: lactose samples were analysed by NMR to investigate the β/α anomer ratio and to monitor whether sucrose had affected this final anomeric composition of freeze-

dried lactose. The NMR results showed that for all freeze-dried sucrose/ lactose mixtures (with a standing time of 4 h of the feed solution), the β/α ratio was 60/40 which meant that sucrose did not affect the final equilibrium of mutarotation of the α- and β- anomers of lactose.

Amorphous sugars were successfully produced by freeze-drying. The confirmation of amorphicity of the produced sugars was investigated by DSC. The integration of the DSC data was performed to obtain enthalpy vs. temperature curves. The glass transition, a second order endothermic transition, is usually seen as a change in the heat capacity as the sample goes from the glass state to the rubber state. Hence, it appears in the DSC thermograms as a step transition rather than a peak. The glass transition was analysed by plotting two tangent lines across the step change corresponding to the glass transition with the point of intersection of both tangents representing the glass transition temperature. The integration of the crystallisation or melting peaks was performed to calculate the amount of heat released or absorbed at a given time by taking the area enclosed by a baseline and the peak between t_0 (the start point or the induction time of crystallisation/thermal event) and t (the end point of the thermal event) (Foubert *et al.*, 2004). The integration of the peak can be attained by various approaches, where the most commonly used ones involve the use of a linear baseline or a sigmoid baseline (Figure 5.7).

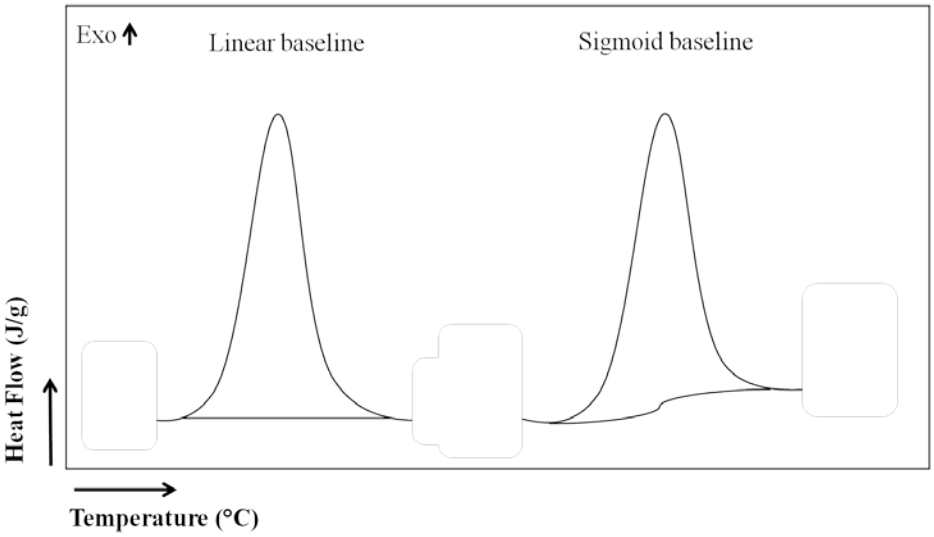

Figure 5.7 DSC integration baselines, showing two modes of integration: Linear baseline and sigmoid baseline.

A sigmoid baseline can be usually obtained by extrapolating the pre- and post- transition baselines at any particular temperature, using the area under the curve to calculate the heat capacity at that particular temperature whereas the linear baseline can be directly selected to join pre- to the post- transition lines using a linear line. A linear baseline is defined as a straight line drawn between the selected start and stop limits. It is used when the baseline varies directly (linearly) with time. The successful application of DSC to characterise amorphous dried sugars has been reported in detail in earlier chapters (chapters 2 & 4). An overlay of six DSC replicates of freeze-dried sucrose in both sealed and pin-holed pans respectively (Figure 5.8) shows that the freeze-dried sucrose is successfully amorphous by showing a T_g, a re-crystallisation peak followed by melting. The DSC scans exhibited high reproducibility with respect to T_g. However, a shift in the onset re-crystallisation temperature and melting/degradation was observed. All the T_g values, onset temperature of re-crystallisation and their enthalpies are summarised in table 5.4.

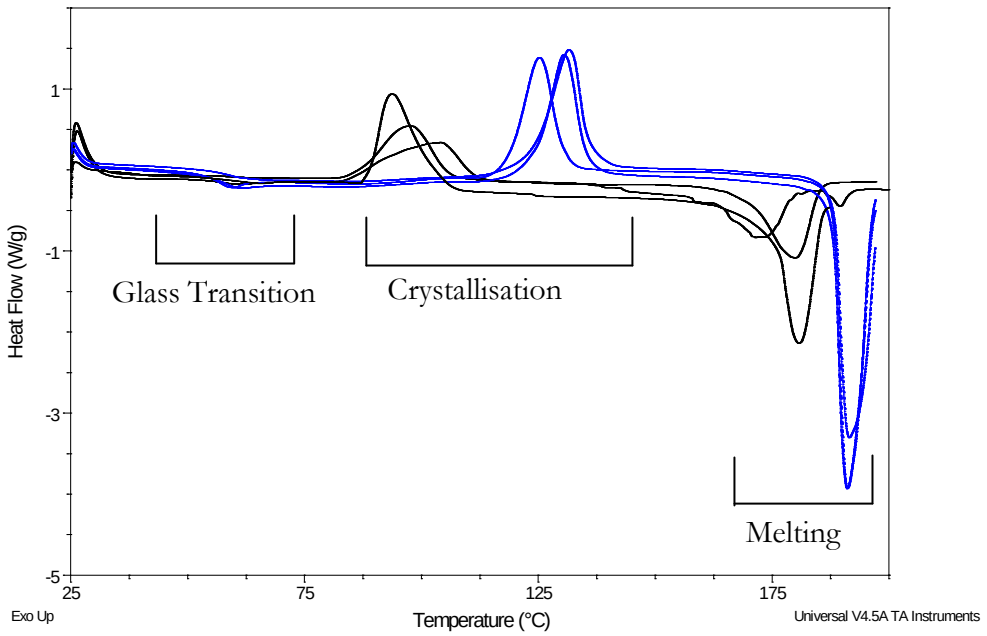

Figure 5.8 An overlay of freeze-dried sucrose in sealed (black) **vs pin-holed** (blue) **pans.**

The DoE was followed and all the samples were run according to the week, day and order number set by the DoE protocol. Hot stage microscopy was used to investigate the amorphism of the freeze-dried sugars (the reasons for running additional hot stage microscopy

experiments have been outlined in chapter 4). The hot stage microscopy images indicated a highly amorphous form of sucrose in the freeze-dried samples. Freeze-dried sucrose appeared highly amorphous because of the absence of any crystalline material in the freeze-dried cake demonstrated by the lack of bifringence. The images collected for freeze-dried sucrose showed no coloured zones at 25 °C; such coloured zone usually appear as result of the interaction between the birefringent anisotropic crystals and the polarized light in an optical microscope as the circular stage is rotated across 360 degrees. So, the highly amorphous nature of freeze-dried sucrose was confirmed as illustrated in figure 5.9 by the absence of any birefringence.

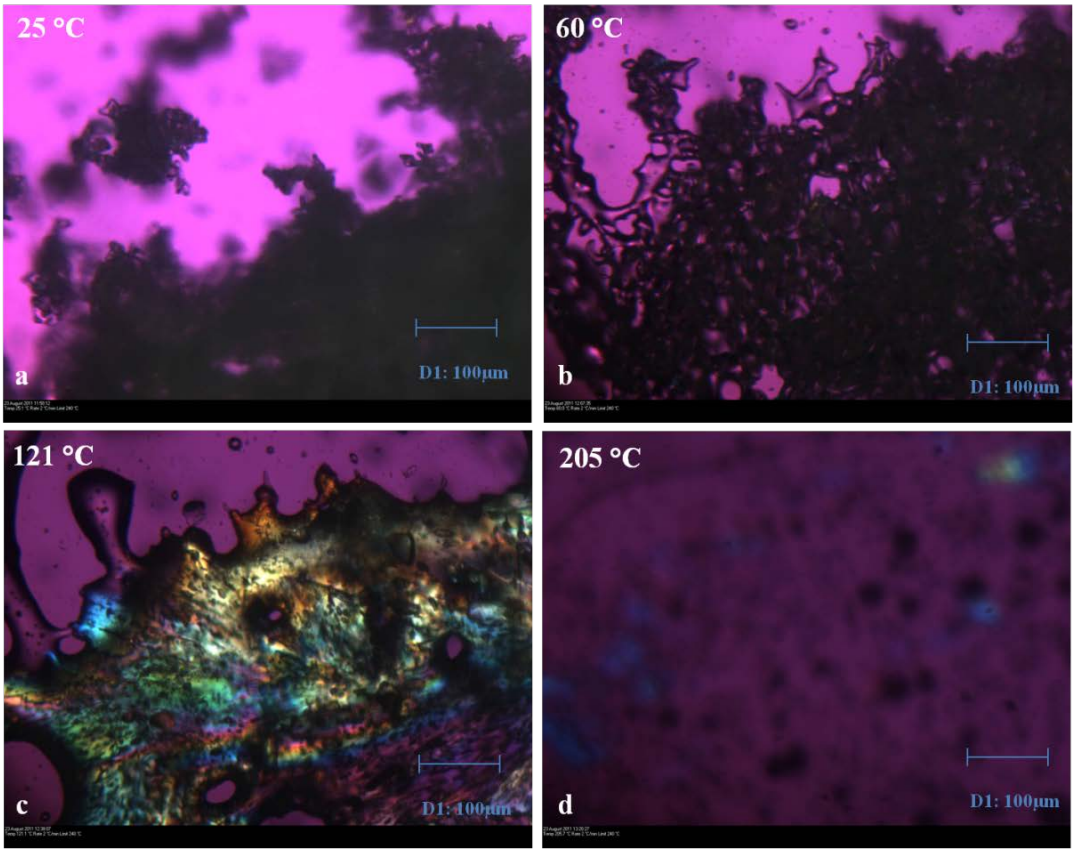

Figure 5.9 Hot Stage microscopy of freeze-dried sucrose (LL) a) 25 °C b) glass transition 60 °C c) crystallisation 121 °C d) melting point 205 °C.

The hot stage microscopy of the freeze-dried sucrose (LL) started at 25 °C which is below the T_g of amorphous sucrose. Upon heating the sample, the amorphous sucrose moved from its glassy state into the rubbery state with a glass transition of 60 °C. The molecules,

thereafter, arranged themselves to obtain a re-crystallisation at 121 °C followed by melting at ~ 204 °C (Figure 5.9).

The DSC thermogram of freeze-dried sucrose also ascertained the success of freeze-drying in producing a wholly amorphous sucrose sample (Figure 5.8). The freeze-dried samples exhibited an initial step change corresponding to the glass transition T_g at ~ 60 °C. This value agrees with lines of experimental evidence reported in literature which state that T_g for sucrose ranges from 50 - 75 ºC (Roe *et al.*, 2005). A comparison between DSC runs of freeze-dried sucrose samples in hermetically sealed pans (black) and in pin-holed pans (blue) shows that both pin-holed and hermetically sealed samples exhibit very similar T_g values (~ 58 - 59 °C) (Figure 5.8). Therefore, both samples were shown to be wholly amorphous with the amount of residual water remaining, being higher in sealed samples than pin-holed samples without affecting the T_g value. To confirm that no water loss was taking place from the hermetically sealed pans (without the pin-hole), the pans were weighed before and after the experiment. It was also decided to compare the crystallisation events for both samples. The reason for selecting the crystallisation peak even though the shapes appeared different was the consistency of the areas (enthalpy) measured in the stability experiments of freeze-dried sucrose reported in chapter 4, the danger of degradation associated with the melt and because of the objective which was to model crystallisation. In contrast to the T_g , which remained constant, the onset temperature of crystallisation of pin-holed samples was lowered by approximately 35 °C compared to the onsets observed for the hermetically sealed samples. Moreover, the enthalpy of crystallisation in pin-holed pans was higher than that of hermetically sealed ones. Thus, the residual water remaining significantly affected the onset temperature of crystallisation. This was demonstrated by lower crystallisation onsets for the samples possessing higher water residual content than those where the residual water content evaporated through the pin-holed pans. The six replicates of the amorphous freeze-dried sucrose were overlaid to investigate the robustness and repeatability of T_g, onset crystallisation temperatures and heat capacities of exotherms and endotherms. For the

pin holed samples, the three runs exhibit a % RSD of 1.9, 2.5 and 8.3 % for the glass transition, onset temperature of re-crystallisation and enthalpies of crystallisation respectively. For the hermetically sealed samples, the three runs exhibit a % RSD of 2.4, 2.5 and 11.6 % for the glass transition, onset temperature of re-crystallisation and enthalpies of crystallisation respectively. These results show high agreement among the different three runs for each of the samples which in turn reflects the repeatability of the DSC response of the freeze-dried sucrose and its wholly amorphous composition.

When the freeze-dried sucrose containing 3% w/w NaCl (HL) was monitored on the hot stage microscope, it showed that the sample passed through the glass transition at 98 °C followed by re-crystallisation at 176 °C. Further heating caused the melting of the sample at 200 °C. Therefore, amorphous sucrose/ 3% w/w NaCl mixture still crystallised but as the hot stage microscopy is a qualitative technique rather than quantitative, it can only monitor the crystallisation without any information about the reduction in the enthalpies of crystallisation (Figure 5.10).

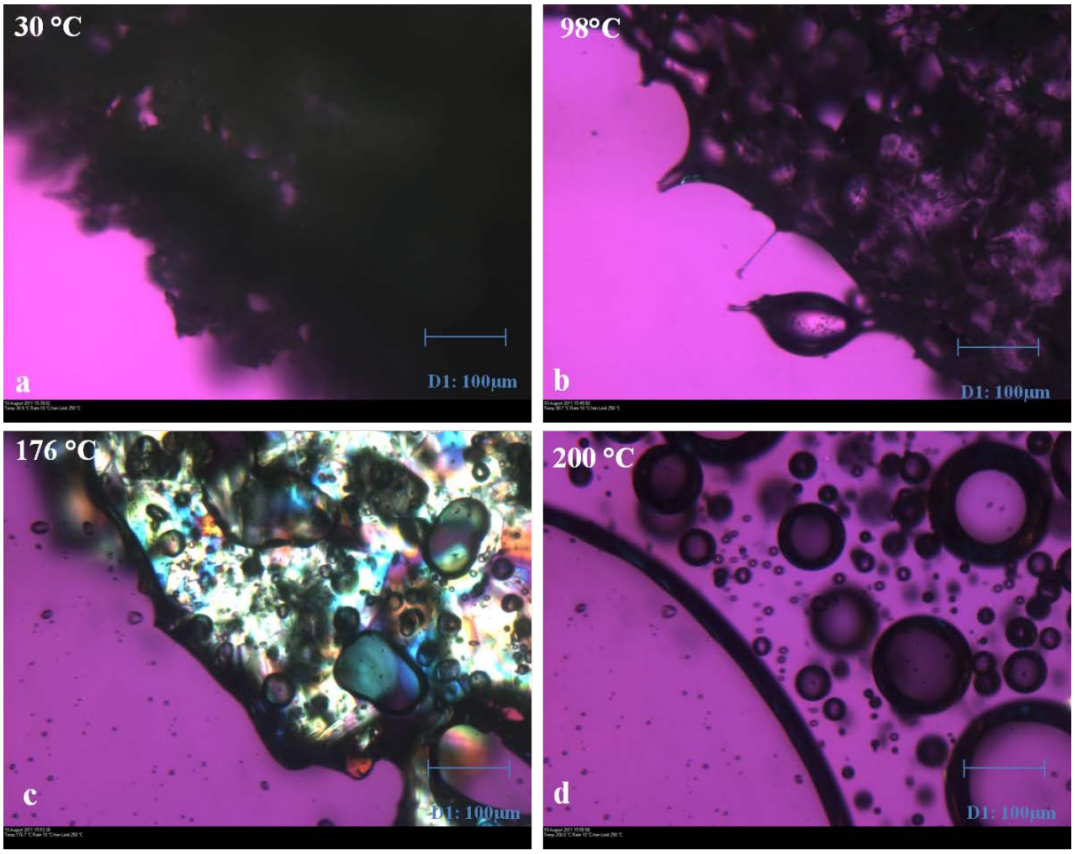

Figure 5.10 Hot Stage microscopy of freeze-dried sucrose containing 3% w/w NaCl a) 30 °C b) glass transition 98 °C c) re-crystallisation 176 °C d) melting point 200 °C.

The six replicates of the amorphous freeze-dried sucrose with 3% w/w NaCl in both hermetically sealed and pin-holed pans were overlaid to investigate the repeatability of the DSC runs and the associated T_g , onset temperature of crystallisation and their enthalpies (Figure 5.11). It was clearly demonstrated that both pin-holed and hermetically sealed samples exhibit the same T_g (~ 52 - 55 °C). However, a shift in the onset temperature of crystallisation was observed showing an earlier re-crystallisation onset temperature for the hermetically sealed pans. For the hermetically sealed samples, the three runs exhibit a % RSD of 2.4, 4.7 and 5.3 % for the glass transition, onset temperature of re-crystallisation and enthalpies of crystallisation respectively, whereas, for the pin holed samples, the three runs exhibit a % RSD of 3.3, 5.3 and 10.7 % for the glass transition, onset temperature of re-crystallisation and enthalpies of

crystallisation respectively. All the values obtained confirm the repeatability of the DSC as a tool that can characterise freeze dried sucrose.

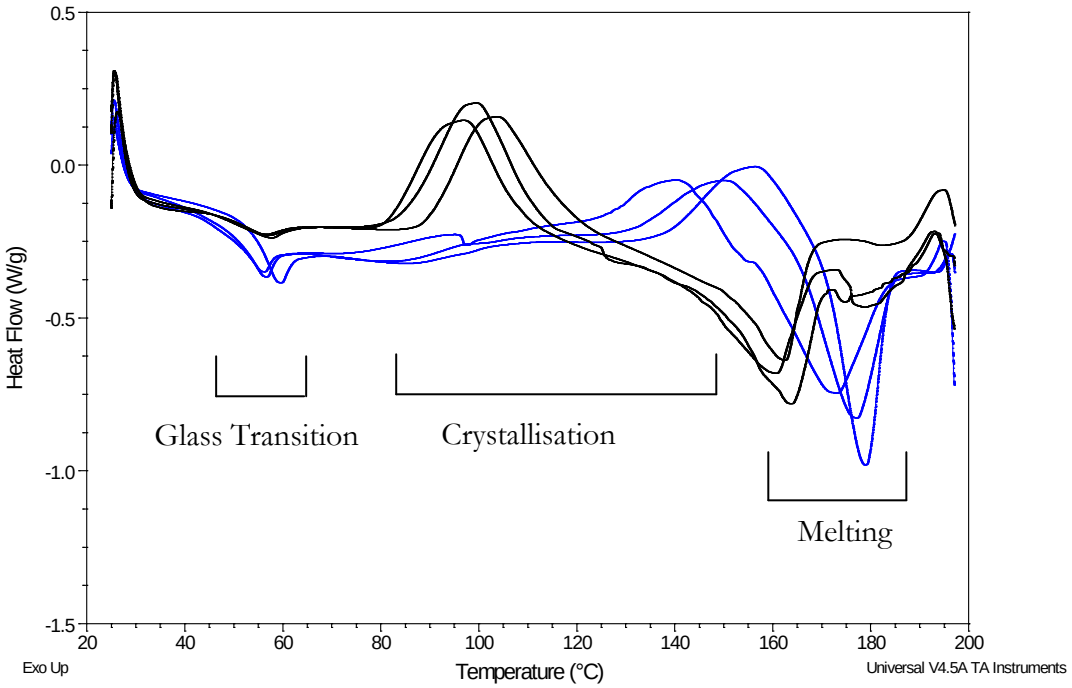

Figure 5.11 An overlay of freeze-dried sucrose containing 3% w/w NaCl, 3 replicates each, in sealed (black) vs. pin holed pans (blue).

An overlay of freeze-dried sucrose (with no minerals or lactose) and freeze-dried sucrose containing 3% w/w NaCl in sealed pans is shown in figure 5.12. By comparing both samples, the freeze-dried sucrose samples exhibited a T_g of 59 °C while the freeze-dried sucrose/3% NaCl showed a T_g of 52 °C. Furthermore, both samples produced very similar onset temperatures of crystallisation (85 - 86 °C) while the enthalpies of re-crystallisation showed a slight difference; the enthalpy of crystallisation of the freeze-dried sucrose was 54.8 J/g while that of the freeze-dried sucrose/3% w/w NaCl was 45.2 J/g.

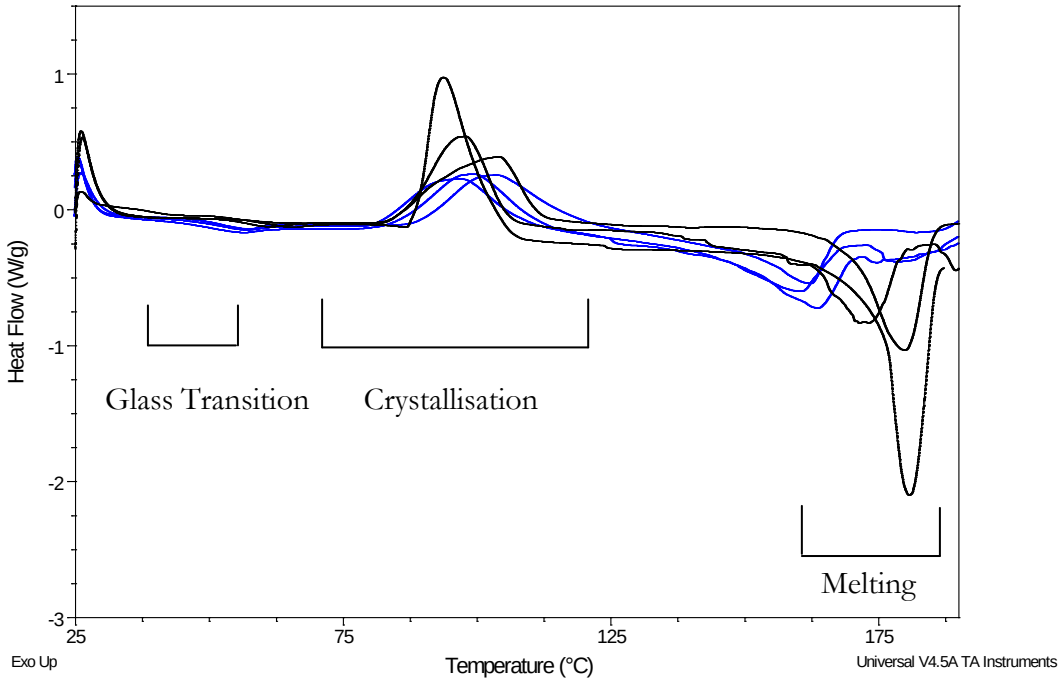

Figure 5.12 An overlay of freeze-dried sucrose (black) **vs. freeze-dried sucrose with 3% w/w NaCl** (blue) **in sealed pans.**

The DSC results indicated that freeze-dried sucrose and freeze-dried sucrose containing 3% w/w NaCl in pin-holed pans showed T_g values of 58.3 °C and 54.8 °C respectively (Figure 5.13). The onset temperatures of crystallisation were 122 °C and 129.5 °C while the enthalpies of re-crystallisation are 72.8 J/g and 35.7 J/g respectively. This means that minerals did not affect the onset temperature of crystallisation as much as the enthalpy of crystallisation itself. Therefore, the crystallisation enthalpy of amorphous sucrose was almost halved in the presence of NaCl, where both samples were run in pin-holed pans i.e. minimal moisture content conditions. Moreover, the onset of melt degradation occurred so early in the presence of NaCl compared to the sucrose only samples. Literature reviews and a detailed interpretation will follow in the discussion section.

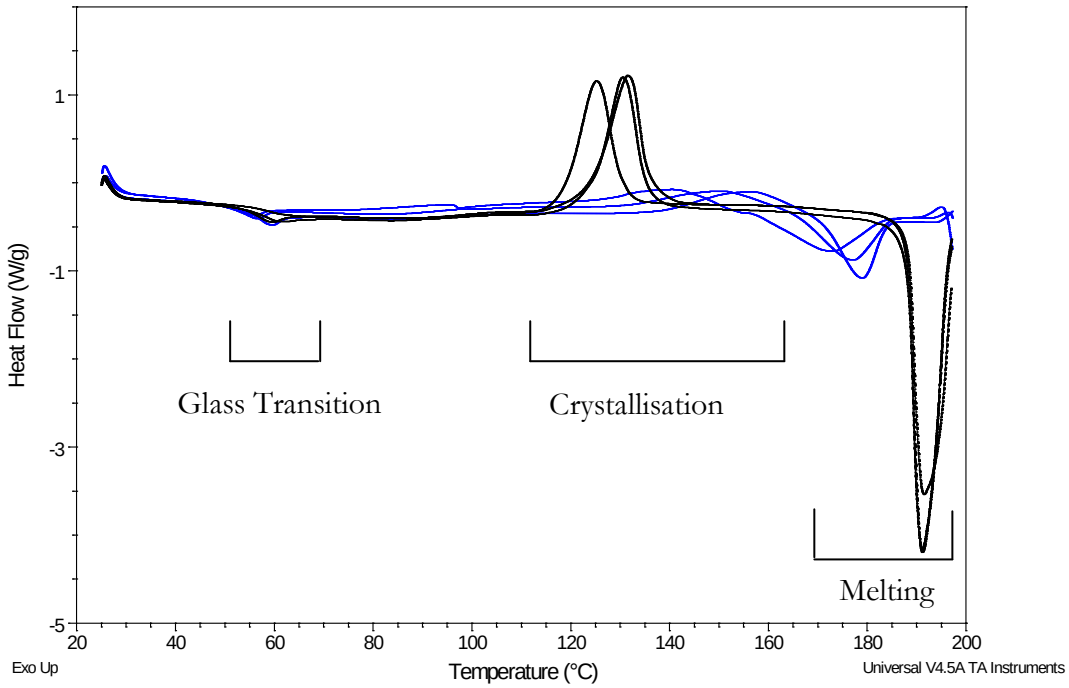

Figure 5.13 An overlay of freeze-dried sucrose (black) **vs. freeze-dried sucrose with 3% w/w NaCl** (blue) **in pin-holed pans.**

Hot stage microscopy of the freeze-dried sucrose/34% w/w lactose (LH) showed a glass transition at 116 °C without any re-crystallisation. Hence, upon heating the sample, it passed from glass transition directly to the liquid state without re-crystallising (Figure 5.14).

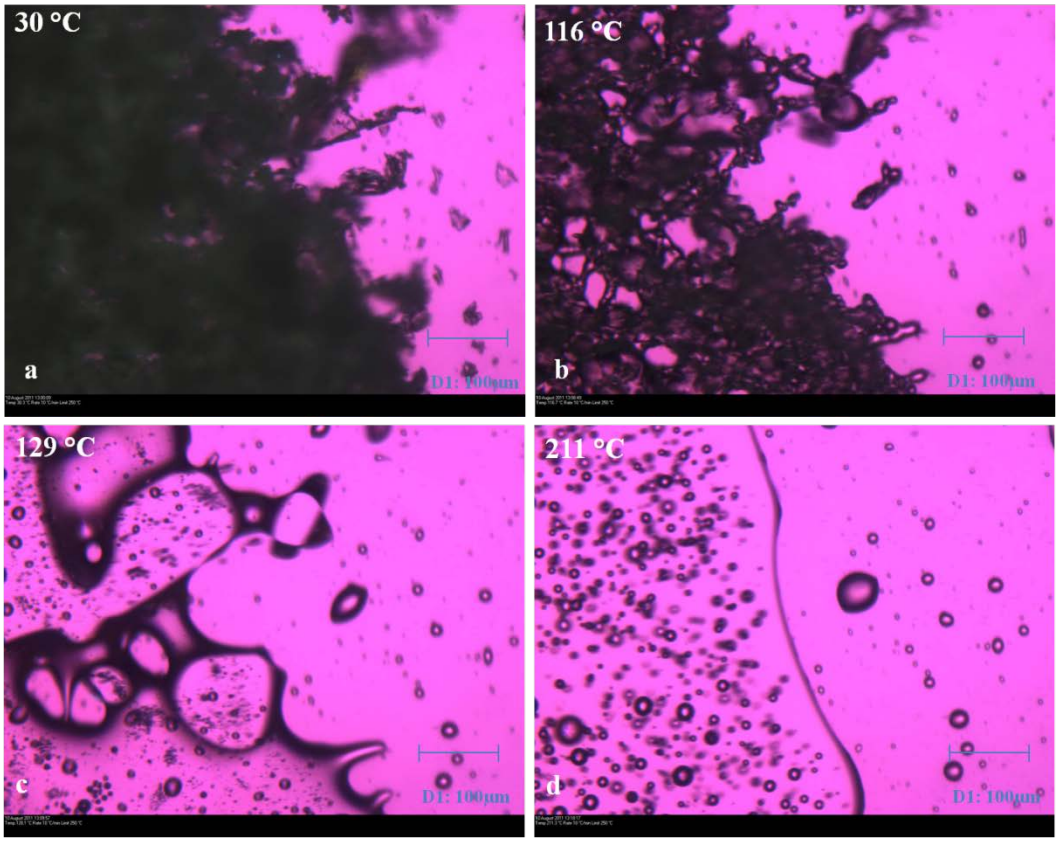

Figure 5.14 Hot stage microscopy of freeze-dried sucrose containing 34% w/w lactose a) 25 °C b) glass transition 116 °C d) liquid state 211 °C.

Overlays of freeze-dried sucrose/17 % w/w lactose and freeze-dried sucrose/1.5% w/w NaCl in sealed and pin-holed pans respectively are shown in figures 5.15 & 5.16. Six replicates were overlaid to investigate the robustness and repeatability of the T_g, onset temperatures of crystallisation and their corresponding enthalpies. For the hermetically sealed samples, the three runs exhibit a mean T_g of 56.4 °C ± 1.4 (± SD, n=3), whereas the pin-holed samples produced a mean T_g of 55.1 °C ± 1.4 (± SD, n=3). However, no re-crystallisation peak was detected for both sugars which suggested that one of the sugars was involved in hindering the re-crystallisation of the other, even at medium levels (Figures 5.15 & 5.16).

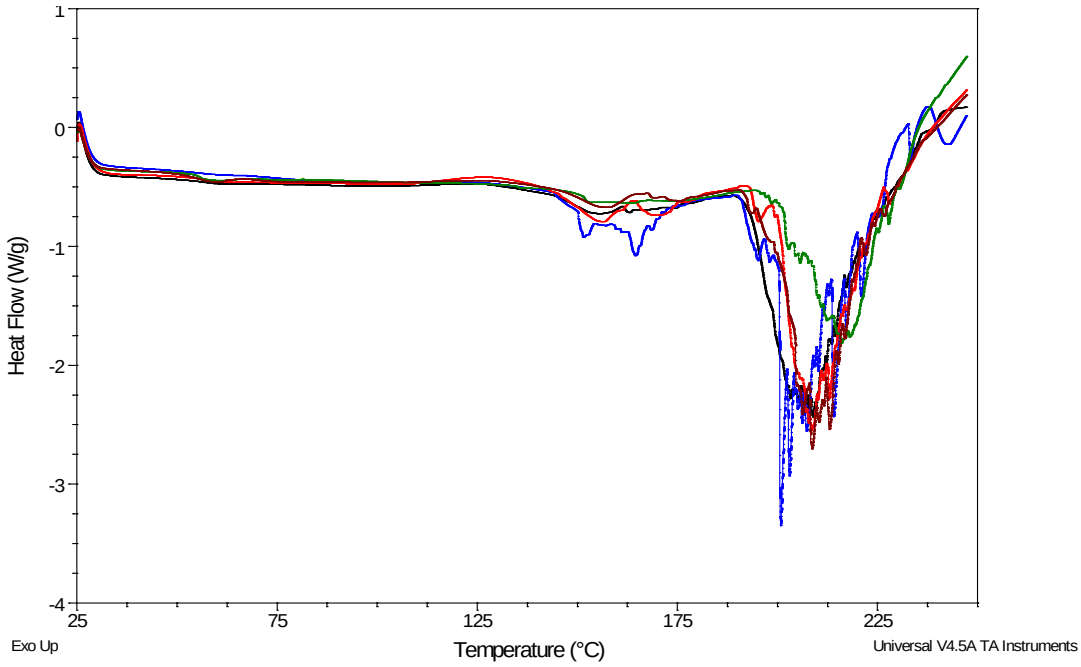

Figure 5.15 An overlay of freeze-dried sucrose containing 17% w/w lactose and 1.5% w/w NaCl in sealed pans.

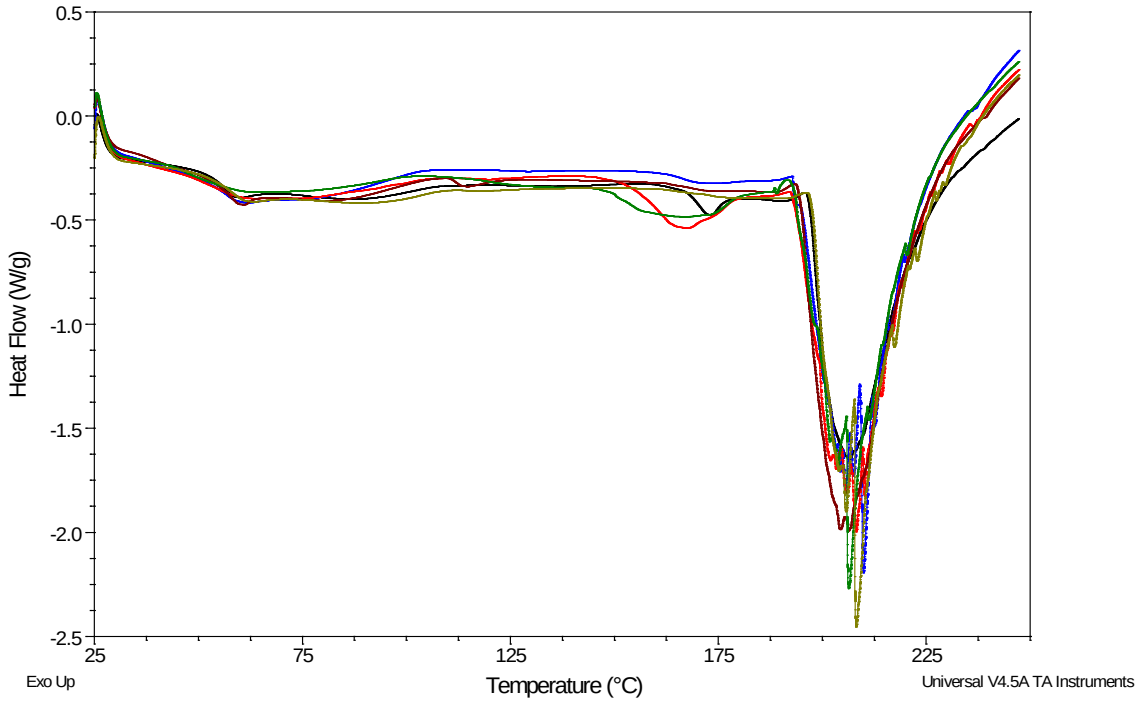

Figure 5.16 An overlay of freeze-dried sucrose containing 17% w/w lactose and 1.5% w/w NaCl in pin-holed pans.

The freeze-dried sucrose/34% w/w lactose/3% w/w NaCl samples possessed a T_g of 57.3°C ± 2.3 (± SD, n = 3) for the pin-holed samples and 56.3°C ± 2.3 (± SD, n = 3) for the hermetically sealed ones whereas no re-crystallisation was observed (Figure 5.17). This can be attributed to either the interaction between the sugars themselves or among the sugars and the mineral content. The DoE data presented and interpreted further in the results sections allowed a more in depth exploration of this observation.

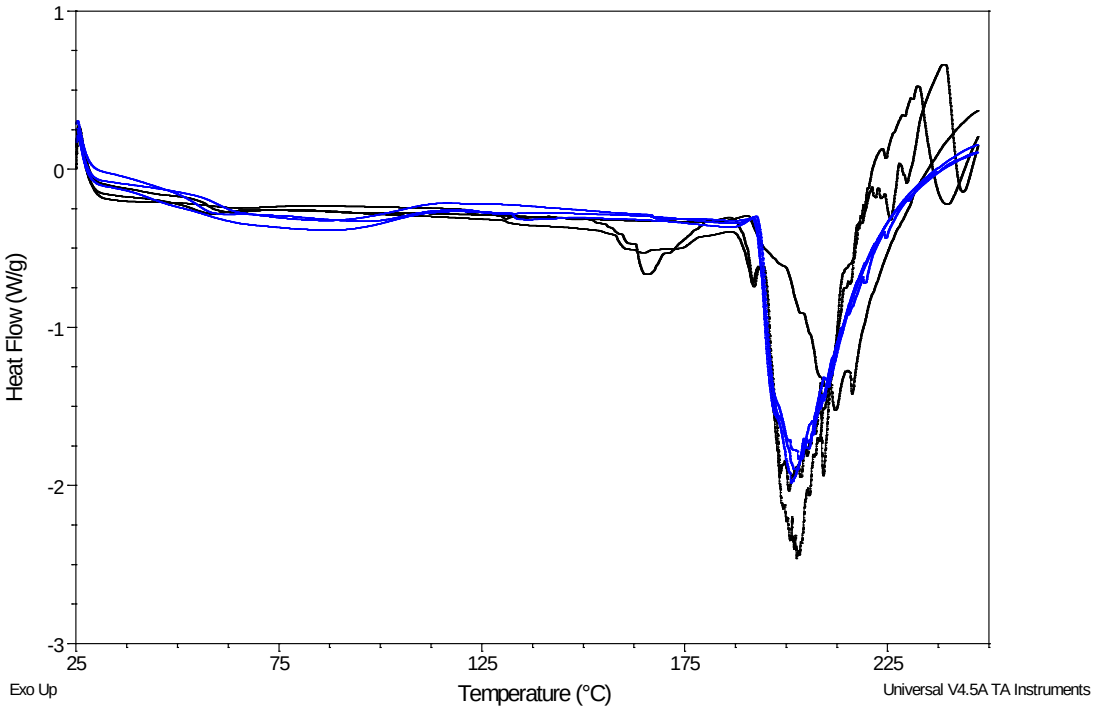

Figure 5.17 An overlay of freeze-dried sucrose containing 34% w/w lactose and 3% w/w NaCl in sealed (black) **vs. pin holed pans** (blue).

The replicates of freeze-dried sucrose/34 % w/w lactose in sealed versus pin-holed pans were overlaid to confirm the repeatability of T_g , onset crystallisation temperatures and enthalpies of re-crystallisation and to compare any differences in the thermal behaviour created due to the variable factors involved (Figure 5.18). For the pin-holed samples, the three runs exhibited a mean T_g of 66.9 °C ± 5.8 % (± SD) and the T_g of the hermetically sealed samples was determined to be 63.5 °C ± 6.7 (± SD). Moreover, no re-crystallisation peak was detected for both types of experiments indicating that both amorphous sucrose and lactose did not crystallise

which might be due to the inhibitory affect of either sugars on the other (Livney *et al.*, 1995) (Figure 5.18).

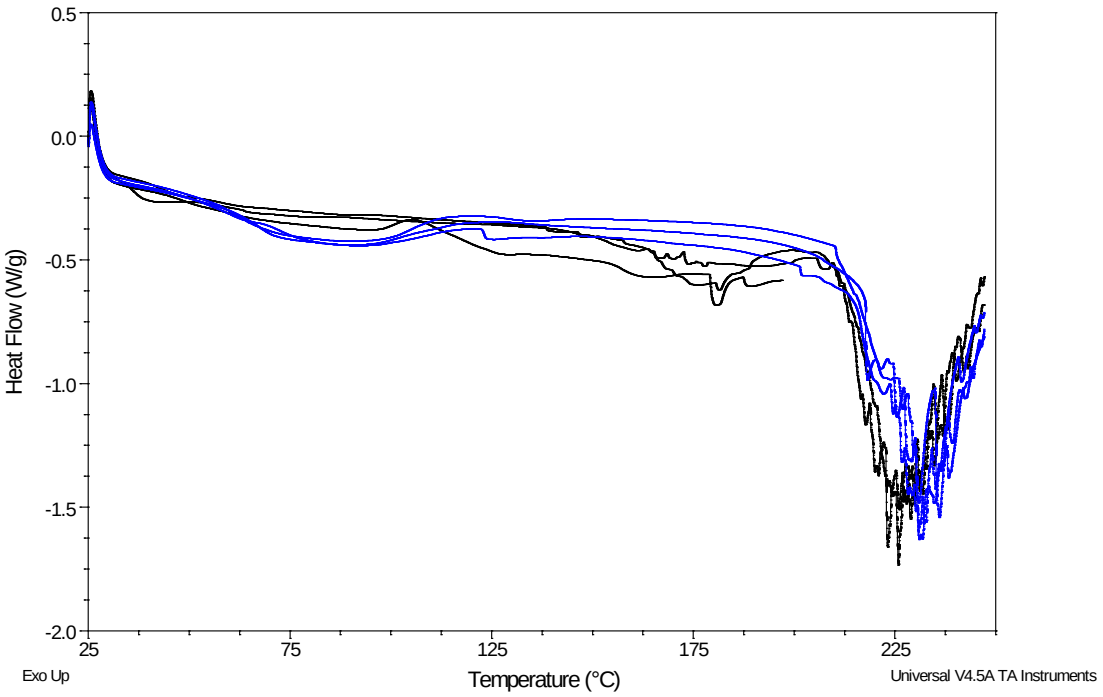

Figure 5.18 An overlay of freeze-dried sucrose containing 34% w/w lactose in sealed (black) **vs. pin holed pans** (blue).

The T_g, onset temperature of re-crystallisation T_{crys} and enthalpy of re-crystallisation are all reported in table 5.4. As seen in the table, the crystallisation onsets and enthalpies of all the freeze-dried sucrose samples containing lactose, were not reported as there was no re-crystallisation taking place.

Table 5.4 A summary of the different freeze-dried sucrose samples with their corresponding T_g, onset temperature of crystallisation and enthalpies of crystallisation.

	T_g °C (± SD, n = 3)	T_{crys} °C (± SD, n = 3)	Enthalpy J/g (± SD, n = 3)
LLU	58.3 ± 1.1	122 ± 3.1	72.8 ± 6.0
LLS	59.2 ± 1.4	86.4 ± 2.2	54.8 ± 6.4
HLU	54.8 ± 1.8	129.5 ± 6.9	35.7 ± 3.8
HLS	52.3 ± 1.3	85.1 ± 4.0	45.2 ± 2.4
HHU	57.3 ± 2.3	-	-
HHS	56.3 ± 2.3	-	-
LHU	62.5 ± 4.5	-	-
LHS	61.0 ± 2.6	-	-
MMU	55.1 ± 4.1	-	-
MMS	56.4 ± 1.4	-	-

The factorial approach design of experiments is widely used in research (Carmona *et al.*, 2005; Bays *et al.*, 2004; Gomez *et al.*, 1984). The model was a standard least squares fitting (with an assumption that the responses were normally distributed) of the independent chosen variables (minerals and lactose concentration, presence/absence of water) against each of the dependant variables (T_g, onset temperature of crystallisation or enthalpy of crystallisation). Therefore, this model was used to obtain the predicted data. Thereafter, the predicted data were compared against the measured values using a simple linear regression. Goodness of fit and mean squared errors were used to check whether the model was capturing the trend in the experimental data (Wissmann, 2008)'.

A whole model of the responses of observed enthalpy for the crystallisation peak was plotted. The model compares the actual results produced to the predicted data. The number of observations is 12, which includes three replicates of each LLU, LLS, HLU and HLS as reported in table 5.4; the r-squared values were calculated and the effects of the 3 input variables on the crystallisation of sucrose were summarised in table 5.5.

Table 5.5 A summary of fit of the heat flow model.

Summary of fit	
r-squared	0.92
r-squared adjusted	0.89
Root mean squared error	4.88
Mean of response	52.19
observations	12

The correlation coefficient (r-squared value) gives an indication of how much of the variability observed in the obtained data is accounted for by the model proposed while the adjusted r-squared value modifies r-squared by taking into account the number of covariates included in the model (Ratner, 2003). The root-mean-square error (RMSE) is often used as an indication of the accuracy of the model fitted to the experimental data. RMSE measures any differences among the values predicted by a model and the actual values observed.

Table 5.6 Effect tests of the heat flow model.

Effect Tests			
Source	**Sum of Squares**	**F Ratio**	**P-value**
Mineral	1652.75	69.15	< 0.0001
Sealing	50.63	2.11	0.1836
Mineral*Sealing	5559.37	23.40	0.0013

With respect to enthalpy, the medium/medium results were excluded from the model as no data was obtained for the re-crystallisation process. The summary of fit of the model exhibited an r-squared value of 0.92 which implies that this is a good fit and indicates that the proposed model fits the experimental data (which means that the measured data are closely following a linear regression trendline). The model showed a p-value < 0.0001 which means that there is a significant difference in the enthalpy of crystallisation (ΔH) with respect to the samples with high versus low mineral content (Tables 5.5 & 5.6). This can be explained as follows: when the statistical test was performed, a null hypothesis was set. The statistical test was conducted at

a significance level of 5 % which is equivalent to 0.05 in decimals. As the p-value is less than 0.05, the null hypothesis was rejected indicating a statistically significant difference in the enthalpies of re-crystallisation ΔH.

The low mineral freeze-dried samples have a mean ΔH = 63.93 J/g while the high mineral freeze-dried samples have a mean ΔH = 40.46 J/g. This indicates that the higher the level of minerals, the lower the measured enthalpy of crystallisation from the amorphous starting forms. An interpretation of either incomplete crystallisation (indicating either the presence of amorphous sugar in the sample even after reaching crystallisation) or crystallisation into another crystalline form with lower heat of fusion, can be made. Furthermore, the method of matching crystallisation peaks and melting may not be applicable as melting in the case of sucrose also incorporates degradation (Lee *et al.*, 2011).

To conclude, the results obtained imply incomplete crystallisation but further work needs to be carried out to investigate the driving factors behind this reduction in enthalpy of crystallisation. One possible experiment which might offer some key elucidations involves grinding physical mixes of sucrose and NaCl and running them together in the DSC. Thereafter, a comparison of the melting peaks could indicate whether the crystal forms are the same.

The impact of sealing versus unsealing on the enthalpy of re-crystallisation was also studied. The statistical test generated a p-value of 0.183 which suggests that sealing has no significant impact on the enthalpy of crystallisation. The high mineral content in sealed versus pin-holed pans reduces the difference between the means which are no longer statistically significant. Therefore, the low mineral content in unsealed samples exhibits greater difference from all other samples as the least square mean is much higher than the others. The interaction between mineral levels and the sealed versus unsealed pans is statistically significant (p-value = 0.001) for enthalpy of crystallisation. The measured mean value of ΔH for low minerals in unsealed pans is 72.8 J/g. The effect of sealing versus unsealing was demonstrated again here as when low levels of minerals are present in sealed pans, the value of ΔH drops from 72.81 J/g to

55 J/g. When samples are sealed, there is no significant difference on enthalpy between high and low mineral content. Samples with high mineral content exhibit no significant difference between sealed and unsealed on the enthalpy of crystallisation. The diffraction of the difference is the same as the low mineral contents whereas, the high versus low mineral contents in unsealed samples show a significant difference on the enthalpy of crystallisation. It is worth noting here that in unsealed pans, the water vapour is replaced in the DSC by N_2 gas.

A model of the responses that compares the actual observed onset temperatures of crystallisation to the predicted data has been plot. Importantly, the r-squared, adjusted r-squared and mean of responses were reported in table 5.7, with an r-squared value of 0.96 meaning that the measured data are highly following a linear regression trendline.

Table 5.7 A summary of fit of the onset temperature of crystallisation model.

Summary of Fit	
r-squared	0.96
r-squared adjusted	0.95
Root mean squared error	4.42
Mean of response	105.75
observations	12

Table 5.8 Effect tests of the onset temperature of crystallisation model.

Effect Tests			
Source	**Sum of Squares**	**F Ratio**	**P-value**
Mineral	28.86	1.47	0.260
Sealing	4789.20	245.00	< 0.0001
Mineral*Sealing	57.24	2.92	0.120

The impact of different variables (mineral content, sucrose:lactose ratio and residual water) on the onset temperature of crystallisation was also studied. The medium/medium results

were excluded from the model as no re-crystallisation occurred and thus no data were collected. The model effect (Table 5.8) shows that the minerals have a p-value of 0.260 implying that the minerals have no significant effect on the onset temperature of crystallisation. The sealing versus unsealing had a p-value which was lower than 0.0001 indicating that there is a statistically significant difference in the onset temperature of crystallisation. Therefore, the sealing versus unsealing factor can affect the onset temperature of crystallisation without influencing the enthalpy of crystallisation. However, the mineral*sealing interaction had no significant impact as its p-value is 0.120.

A model of the responses of the actual measured T_g for all of the freeze-dried sucrose samples was plotted (for the medium levels). The r-squared values, r-square adjusted, mean of responses were determined (Table 5.9).

Table 5.9 A summary of fit of the glass transition model (medium only).

Summary of Fit	
r-squared	0.04
r-squared adjusted	-0.04
Root mean squared error	3.04
Mean of response	55.78
observations	12

The effect of the input variable on the crystallisation of freeze-dried sucrose was tabulated (Table 5.10). A p-value of 0.500 proves the absence of any statistically significant difference, meaning that sealing had no impact on the T_g of the freeze-dried sucrose at the medium level of mineral and lactose content.

Table 5.10 Effect tests of the glass transition model (medium only).

Effect Tests			
Source	**Sum of Squares**	**F Ratio**	**Prob > F**
Sealing	4.55	0.48	0.500

A model of the responses of the actual measured T_g for all of the freeze-dried sucrose samples was plotted (no medium levels included). The r-squared values, r-square adjusted, mean of responses are all listed in table 5.11.

Table 5.11 A summary of fit for the glass transition model (no medium).

Summary of Fit	
r-squared	0.17
r-squared adjusted	-0.17
Root mean square error	20.67
Mean of response	50.76
observations	21

The effects of the three input variables were studied and the statistical data are tabulated (Table 5.12).

Table 5.12 Effect tests of the glass transition model (no medium).

Effect Tests			
Source	**Sum of Squares**	**F Ratio**	**P-value**
Mineral	15.54	0.03	0.85
Ratio	87.48	0.20	0.65
Sealing	215.39	0.50	0.48
Mineral*Ratio	105.61	0.24	0.62
Mineral*Sealing	0.10	0.0002	0.98
Ratio*Sealing	234.61	0.54	0.47

With respect to T_g, both medium and no medium analysis showed that all the factors including minerals, sucrose:lactose ratio, sealing, mineral*ratio interaction, mineral*sealing interaction and ratio*sealing interaction had no statistical significant difference or effects on the T_g as all p-values of the aforementioned parameters were greater than 0.05 (Tables 5.12 & 5.13).

As mentioned earlier in the results section, hot stage microscopy was implemented as an auxiliary tool to support DSC results confirming the amorphicity of the freeze-dried sucrose mixtures. Furthermore, it was used as a confirmation tool to investigate and monitor the behaviour of the freeze-dried sucrose mixtures upon heating up the sample (Figures 5.9, 5.10 & 5.14). The T_g and temperature of re-crystallisation T_{crys} of the LL sample were exactly matching the results obtained from the DSC yet the melting point of the sample on the hot stage microscope was higher by ~ 15 °C (Figure 5.9). However, the T_g, temperature of re-crystallisation and melting for the freeze-dried sucrose/3 % w/w NaCl (HL) showed significant delays from those recorded by DSC. The hot stage microscope reported T_g, temperature of re-crystallisation and melting of 98 °C, 176 °C, and 200 °C respectively while the corresponding measurements on the DSC were 55 °C, 130 °C and 180 °C respectively (Figure 5.10). Also, the T_g of the freeze-dried sucrose/34 % w/w lactose LH (Figure 5.14) was delayed on the hot stage microscope by ~ 50 °C. This issue was previously addressed in chapter 4. Based on the investigation made, this delay in temperature might be due to the thermal lag between the hot stage (generating the heat) and the actual slide, or the particle size of the sample (as grinding some samples produced data that were closer to those recorded for the DSC) or the uncontrolled environment of the sample, as in hot stage microscope the sample was analysed in open air whereas in DSC, all samples were run under N_2.

5.4 Discussion

The aim of this study was to measure the crystallisation of sucrose in the presence of lactose, minerals and water vapour. The model system studied encorporated sucrose and lactose as the major components due to the paucity of the understanding of sugars and their crystallisation behaviour in crumb; thus, the model system consisted mainly of sugars i.e. sucrose and lactose, with the minor components as minerals and water vapour.

The research objectives involved establishing a simple model which represented the forms or the stages that sucrose might go through in the crumb reactor. The second objective was to apply a thermo-analytical method which could monitor the thermal changes that sucrose may undergo in the reactor during the crumb cooking process. The third objective was to design a set of experiments that would determine the impact of the individual components and highlight any interactions between them.

During the cooking of a chocolate crumb, in most systems the sugars and milk are heated in a mixture containing approximately 25% water. As the mixture is heated, the water is drawn off and it is thought that all the sugars are fully solubilised before re-crystallisation commences (Elleman, C.J., personal communication). As water is removed by vacuum, the system becomes supersatured and this is relieved by the crystallisation of the sugars. Since this type of system is impossible to reproduce in DSC pans where thermal events can be monitored, it was decided that a model system would be to look at the re-crystallisation of both sucrose and lactose from freeze-dried preparations. The ratios of sugars used in this reaction were close to those found in a standard crumb recipe. The starting points were from the amorphous forms of both sugars, with known purity, and the aim was to monitor and measure their crystallisation behaviours.

The hypothesis being tested here is that sucrose in a crumb behaves differently with respect to crystallisation in the presence of different levels of minerals, water and lactose content.

The crumb manufacturing process was extensively discussed in the introduction of this chapter. The first approach for monitoring the sugar behaviour that was considered was to heat the crystalline sucrose in the DSC until melting and then quench cooling to produce its amorphous form. Lee and co-workers (2011) reported that the melting of sucrose is a decomposition reaction. Therefore, a concern about the thermal degradation of sucrose and the purity of sucrose after melting was raised. Thus this approach was excluded.

The alternative approach was to start with a freeze-dried amorphous sucrose of known purity (as detailed in chapter 4). The purity of the starting sugar materials, sucrose and lactose, was determined in chapters 2 & 4 where the freeze-dried sucrose was found to be pure and void of any invert sugars and the β/α anomeric content of freeze-dried lactose was also determined. DoE involved the impact of many variables on the crystallisation of sucrose so if the purity of sugars was not determined, this will create an extra set of variables where the impact of the intended parameters might be lost.

The selection of the amorphous forms of the sugars as a starting point can perfectly aid in monitoring their re-crystallisation behaviour according to the variable factors involved. Correspondingly, this will provide a better understanding of the impact of the three different variables chosen which are mineral content (NaCl), residual water content (hermetically sealed versus pin-holed pans) and lactose content on sucrose, with any possible interactions. The model system chosen for studying the thermal changes associated with crystallisation in the presence of other sugars and minerals is considered appropriate as a way of controlling the presence/absence of the selected variables. This is because it allows the control of temperature whilst monitoring phase behaviour of samples. It was seen before (in chapters 2 & 4) that a single system of lactose and/or sucrose showed variances in T_g while the enthalpy of crystallisation was relatively stable. That's why it is believed that the area (enthalpy) of crystallisation is a very important parameter to monitor.

Freeze-drying of sucrose mixtures with minerals and lactose, as described in the methods section, was performed. Hot stage microscopy and DSC were both utilised to confirm the amorphous nature of the freeze-dried sucrose.

Hot stage microscopy (Figures 5.9, 5.10, & 5.14) showed no birefringence in the images captured at 25 - 30 °C, indicating the absence of any crystals. The samples were then heated at a rate of 2 °C/min where they passed from the glassy state into the rubbery state indicating a glass transition. Further heating caused a rearrangement of the molecules and re-crystallisation

occurred. This was exhibited by colourful images produced from the birefringence due to the presence of crystals. Upon more heating, the whole sample melted and transformed to the liquid state. Therefore, hot stage microscopy indicated the presence of amorphous material in the freeze-dried sucose mixtures.

The generated freeze-dried fluffy cake was further characterised by DSC. The DSC thermograms revealed a step change referring to the glass transition. Upon heating the sample, the mobility of the molecules started to increase, thus re-arranging themselves and re-crystallising, followed by melting of the crystals formed. This implies that DSC was successfully able to assess the freeze-dried sugars and prove the presence of amorphous material.

A method for producing amorphous sucrose in the presence of lactose and NaCl was successfully developed. The DSC was run in both open and closed pans, the closed pans retaining vapour and the open pans allowing residual water to escape during the DSC experiments; this provided a model system that mimicked the difference between having a full vacuum applied during cooking and the situation where the pressure was increased. Therefore, the first objective of the work reported in this chapter was met.

It was proved in the previous chapters (chapters 2 & 4) that DSC is able to detect and measure the glass transition, crystallisation and melting peaks of both amorphous sucrose and lactose. Therefore, and based on the previous chapters, the thermal profile of the DSC method was chosen to start at 25 °C at a 10 °C/min heating rate until 200/250 °C to cover the heating range of the reactor which normally starts at room temperature and heats up to 90 - 124 °C. Thus, this DSC method will be able to monitor the as-produced amorphous sugars and their re-crystallisation at higher temperatures until they reached melting.

The DoE was structured to set the chronological order of the experiments based on the randomization approach which enhances the precision of the experiments and reduces any possible bias. The DoE was applied to save the "one change at a time" and to be able to study the effects of all possible interactions in a less number of combinations.

The freeze-dried sucrose samples showed a water content of 1.6 % w/w, which matches the water content of the crumb ~ 1.5 % w/w; whereas, the freeze-dried sucrose/3% w/w NaCl possess a water content of 2.3 % w/w. The amorphous sucrose/3% NaCl exhibited higher water content than the amorphous sucrose. This implies that the presence of salt plays a pivotal role in trapping more water within the freeze-dried sucrose cake. The ability of minerals to sequester water was reported by Santagapita *et al.* (2008) and Chen *at al.* (2005). They attributed this affinity of salts for water to water-cation interactions and it is assumed that this mechanism brought about the doubling of water content for the freeze-dried sucrose samples prepared in the presence of NaCl and reported in this thesis. Such an observation has a potential impact on the crumb and different forms of milk, which will have different amounts of minerals available for interaction with sucrose.

Both amorphous sucrose and sucrose/3 %w/w NaCl were analysed by DSC. Freeze-dried sucrose showed a T_g value of 59 °C while the sucrose/3% NaCl had a T_g value of 52 °C. Both values can be considered very similar with a slight drop for the samples containing 3% NaCl. Literature reported that the addition of salts did not affect the glass transition of amorphous sugars (Mazzobre *et al.*, 1999, Longinotti *et al.*, 2002, Ohtake *et al.*, 2004) where it was reported that increasing the potassium ions concentration by 0.11 in an amorphous sugar-phosphate mixture at a constant pH of 7, did not alter the T_g of sucrose (59°C).

It should be noted here that in the thesis, several batches of freeze-dried sucrose have been reported. However, many had been prepared and examples of which are described in table 6.1.

Table 6.1 A comparison of 4 batches of freeze-dried sucrose where batch 1 from chapter 4 while batch 4 from chapter 5.

	Batch 1	**Batch 2**	**Batch 3**	**Batch 4**
T_g **°C**	47.2 ± 0.8	55.1 ± 0.5	63.6 ± 0.5	58.3 ± 1.1
T_{crys} **°C**	116.6 ± 2.5	117.3 ± 1.9	113.6 ± 4.2	122 ± 3.1
Enthalpy of crystallisation (J/g)	79.0 ± 0.5	74.9 ± 3.2	74.5 ± 9.3	72.8 ± 6.0
DSC used	DSC 2920	DSC 2920	DSC Q20	DSC Q20
Date of preparation	Nov, 2009	Feb, 2010	May, 2011	July, 2011
Water content % w/w	1.2 (TGA)	-	-	1.6 (KarlFisher)

It can be seen that whilst there is a variance in the reported T_g values (47 - 63 °C), the enthalpy of crystallisation values were relatively repeatable (73 – 79 J/g). The hypothesis behind such a variation in the T_g value might be due to differences in the water content of the samples or in the thermal profile of water loss from the sample i.e. different sublimation rates. However, this was not an issue as DoE has proven that T_g did not have an impact on the re-crystallisation of freeze-dried sucrose mixtures because T_g values were less sensitive to composition and showed a high variance. However, as the enthalpies of crystallisation are more relevant to the aim of this study and were more repeatable, the enthalpy of crystallisation was considered a better indication of stability.

The presence of minerals affected the crystallisation enthalpy of amorphous sucrose yet without affecting the onset temperature of crystallisation. This was demonstrated in a smaller observed enthalpy of crystallisation corresponding to the amorphous sucrose/3% w/w NaCl. This might be due to the NaCl salt that is inhibiting the crystallisation process of the sugars. Hartel (1991) reported that the addition of any impurities to a sugar system often influences either solubility or melting point which in turn impacts the crystallisation process. It was also illustrated by Telang and colleagues (2003) that salts, including NaCl were found to be significantly effective in inhibiting the crystallisation of mannitol from frozen systems. The inhibitory effect of NaCl was observed at concentrations ranging from as low as 0.5% w/v to 5 % w/v where the higher the NaCl content, the harder it was for Mannitol to crystallise. It was

also shown by Santagapita *et al.* (2008) that the presence of minerals can influence the crystallisation temperatures and enthalpies of sugars. This inhibitory effect of crystallisation induced by NaCl can be assumed to be due to the tendency of NaCl to keep the water in hydration shells surrounding its ions thus preventing it from contributing to the re-crystallisation process. The mechanism involved is based on the interaction of the mineral ions with water which is attributed to the charge densities as well as the electrostatic forces that hold the water to the mineral ions (water dipoles interacting with ions) and hydrogen bonding (water interacting with the neighbouring water) (Hribar *et al.*, 2002; Her *et al.*, 1995).

Crystallisation takes place during crumb manufacture. This often occurs under reduced pressure (which drives off water from the mixture). Thus, residual water content was chosen as the second variable to be investigated. Hermetically sealed pans versus pin-holed pans were used to mimic high residual water content versus low residual water content respectively. Both thermograms of freeze-dried sucrose in hermetically sealed pans versus pin-holed pans showed the same T_g ~ 60 °C. However, hermetically sealed amorphous sucrose samples showed an earlier onset of crystallisation than the pin-holed ones. This can be contributed to the sealing effect which captures water in the sample while the pin-holed pans allow water to evaporate, which means that the higher the moisture content in the pans, the earlier the crystallisation takes place. This can be attributed to the mobility of the molecules which increases in the presence of water and consequently less energy is needed for the sucrose molecules to diffuse and orientate to join the forming and growing crystals. This theory is supported in the literature by Labuza (2004) who reported that an increase in moisture content will increase the mobility of the molecules thus the glass enters the rubbery state faster which leads to earlier crystallisation (Labuza 2004). Makower *et al.* (1956) showed that exposing amorphous sucrose to high humidity increased the absorbed water content of the samples and thus initiated crystallisation.

It was decided to rely on observing the crystallisation peaks rather than the melting peaks of the samples. This is because it was observed that in the samples containing NaCl with water

vapour trapped in (hermetically sealed pans), the samples exhibited a melting peak with an earlier onset of melting and with lower enthalpy. This might be due to the chemical degradation happening earlier and/or the colligative effect of NaCl on melting.

A significant observation was that the T_g value was not affected by the addition of NaCl to the model system despite that NaCl complexes with water and thus increases the water content of the sample. This suggests that NaCl was not initially in an amorphous phase i.e. in a separate phase from amorphous sucrose, otherwise T_g would have changed (Her *et al.*, 1995). Upon heating, and once the T_g is passed, sucrose becomes less viscous and consequently NaCl starts mixing with the system in one phase and thus increases the mobility inducing an earlier crystallisation; however, this crystallisation is incomplete as NaCl still plays its inhibitory role in preventing crystallisation as discussed in an earlier section.

As milk (containing lactose) is one of the main constituents of chocolate crumb, the impact of lactose on crystallisation of sucrose was selected as the third variable to be studied. Amorphous sucrose was prepared at three different sucrose: lactose concentrations which were 0% w/w lactose, 17% w/w lactose and 34% w/w lactose. The thermograms of amorphous sucrose at both levels of lactose (17% w/w and 34% w/w) showed no re-crystallisation at all. Thus it appears that both sugars are inhibiting the crystallisation of the other. Kedward (1998) established that crystallisation rates are lower in mixed sugar system of sucrose and lactose than in a single sugar system as the crystal growth and diffusion of lactose into solution were inhibited by the presence of sucrose. Labuza (2006) described the impact of adding trehalose on the crystallisation of amorphous sucrose systems. Labuza (2006) explained that the shelf-life of pure sucrose systems can be very short. Amorphous sucrose systems held above the glass transition temperature will collapse and crystallise where several lines of experimental evidence also showed that by adding a small percentage of another type of sugar to sucrose, the shelf-life of amorphous systems can be extended by slowing crystallisation. The same investigation conducted by Labuza (2006) also demonstrated that raffinose slows down the crystallisation rate

of sucrose in low moisture amorphous state systems. Moreover, trehalose was found to interrupt the crystallisation of sucrose due to its higher glass transition compared to sucrose and the mechanism of inhibition can be explained by the attachment of the glucose units of one sugar to the major planar growing surface of the crystal. This can also apply to lactose which possesses a T_g that is higher than that of sucrose. Leinen (2006) also concluded that raffinose decreases the rate of crystallisation of an amorphous system held above its T_g and the actual mechanism is thought to involve the attachment of the sucrose part of raffinose on the major growing face of the already formed sucrose crystal. In another study conducted by Hartel (1991) it was shown that the presence of lactose and sucrose in a mixture can influence the crystallisation of each sugar. Nickerson *et al.* (1972) studied the crystallisation of sucrose/lactose mixtures at different ratios ranging from 100 % w/w sucrose to 50 % w/w sucrose. They reported that only sucrose crystallised out of the 100:0 and 90:10 sucrose: lactose solutions noting that the rate of crystallisation decreased as the lactose content increased. Moreover, Livney (1995) detailed that sucrose may inhibit the nucleation process of lactose, thus, promoting supersaturation.

Arvanito *et al.* (2004) studied the isothermal crystallisation kinetics of glassy lactose/sucrose mixtures where they observed that a decrease in the crystallisation velocity constants (*k*) was related to the increase in the lactose content of lactose/sucrose mixtures. It was reported by Anzai *et al.* (2011) that the crystallisation of lactose and trehalose in corn starch-disaccharide amorphous mixtures occurred at a_w = 0.75 but sucrose did not. It was harder for sucrose to crystallise in the mixture even at high a_w ($a_w > 0.75$). Such results imply that the interactions between sucrose, starch and water molecules are different from other disaccharides. Kedward (1998) also showed that sucrose-free crumb appeared to contain more crystalline α-lactose monohydrate then standard crumb (around 10% w/w), which suggested that sucrose has an inhibitory effect on the crystallisation of α-lactose monohydrate in a crumb.

Furthermore, according to the same study, the FTIR spectrum of a standard chocolate crumb was found to be very similar to sucrose in the region of 1050-800 cm^{-1}. However, a

shoulder was detected on one of the peaks at around 877 cm^{-1} and a small peak was found at around 835 cm^{-1}, both of which were attributed to crystalline lactose. In developing the quantification method, for simplification it was assumed that α-lactose monohydrate and β-lactose were the only lactose crystals formed in crumb. The chocolate was seeded with lactose and conched for 5 hours and then the viscosity was measured at regular intervals during conching. The conclusion was that lactose seeded chocolate gave a lower viscosity results than standard chocolate when conched for the same length of time. This suggested that seeding with lactose improved the performance of crumb in chocolate manufacture (Kedward, 1998). The opposite case of sucrose addition to amorphous lactose was studied by Thomsen *et al.* (2006) who reported a slightly lowered T_g and a 4 fold increase in the induction time for crystallisation, upon adding sucrose to amorphous lactose.

The mechanism of inhibition of the crystallisation process for sucrose was attributed to the nucleation phenomena in amorphous sucrose (van Scoik *et al.*, 1990). van Scoik referred the inhibition of the crystallisation of sucrose to the nature of the additives which altered the ability of water to act as a solvent for sucrose. In general, water (in this chapter, it is the residual water content of the system) plays a key role in the crystallisation process of sucrose as it enhances the movement and collisions of sucrose molecules necessary to build up the critical nucleus. Therefore, the more hydrophilic the additive is, the stronger its tendency to hydrogen bond with any water present in the system (van Scoik *et al.*, 1999). In general terms, impurities can influence the solubility of the crystallising compounds and thus impurities will have a major influence on both nucleation and crystal growth (Martins *et al.*, 2011).

Therefore, as there is a limited amount of residual water in freeze-dried systems, it aids the crystallisation of sucrose from its amorphous form. The addition of any molecule, NaCl or lactose, which has an affinity for water, will reduce the amount of available water and thus will in turn slow or even inhibit the crystallisation of sucrose. However, lactose proved to have an extra

inhibitory mechanism which does not allow any re-crystallisation due to the interaction with the crystal face mentioned earlier in this section.

Therefore, the study of the impact of minerals, lactose and water vapour on the crystallisation of sucrose, showed that the presence of minerals reduces crystallisation which results in an increase in the amorphous content of the material. This probably accounts for the power increases mentioned earlier during processing. The fact that crystallisation occurs at a lower temperature in closed pans supports the observations made in the pilot plant that increasing the pressure during phase change reduces the power load during the crystallisation (Harris 2012, personal communication) both with and without minerals.

5.5 Conclusion

It was concluded that a shortage of water during crystallisation in the head space of the pan necessitates a higher crystallisation temperature T_{crys}. This has explained the empirical observation from pilot scale crumb making that increasing pressure which will increase H_2O in the head space, improves crystallisation during phase change. Minerals also have an impact on the recipe by reducing the rate of crystallisation at a concentration of 3% w/w NaCl. The presence of lactose in the recipe causes an interaction between lactose and sucrose, also inhibiting the crystallisation of both sugars. Therefore, the impact of different variables and interactions possible while processing a chocolate crumb has been clearly addressed and understood. DoE has also proven to be a very efficient methodology, by saving time and resources, to investigate the correlations that may exist among different variables.

Chapter six - General discussion

6.1 Discussion

Although many research papers have been published on the crystallisation within chocolate and its impact on the chocolate properties, the data reported concerning the chocolate sugars and their purity remains very limited (Ziegleder *et al.*, 1990; Loisel *et al.*, 1998; Tietz *et al.*, 2000; Sato, 2001; Attaie *et al.*, 2003; Foubert *et al.*, 2004; Vereecken *et al.*, 2007). Chocolate sugars, in particular sucrose and lactose, have been researched extensively in both confectionary and pharmaceutical sciences especially concerning crystallisation from the amorphous state (Bhugra *et al.*, 2007; Ibach *et al.*, 2007; Omar *et al.*, 2007; Leinen *et al.*, 2006; Haque *et al.*, 2005; Miao *et al.*, 2005; Shamblin *et al.*, 2005; Newell *et al.*, 2001; Jouppila *et al.*, 1997; Shalaev *et al.*, 1996; Saleki-Gerhardt *et al.*, 1994; Roos *et al.*, 1991; Lai *et al.*, 1990; Islesias *et al.*, 1978; Makower *et al.*, 1956). However, as discussed in chapters 2, 3 & 4, many of these publications have not considered the purity of the sugars with respect to epimerisation and inversion. Thus, in order to address issues concerning crystallisation, the study presented here was successful in characterising the physical and chemical composition of both lactose and sucrose.

Lactose has two anomers α- and β- which differ in their physical properties (McSweeney *et al.*, 2009; Drapier-Beche, 1999). A contributing factor to the wide ranging of anomeric compositions of amorphous lactose reported in the literature (Ramos *et al.*, 2005; Roetman *et al.*, 1975; Chidavaenzi *et al.*, 1997; Buckton *et al.*, 2002; Listiohadi *et al.*, 2009) is a poor appreciation of the epimerisation equilibrium within the feed solutions prior to initiation of the drying processes, for example freeze- and spray-drying, which are typically used to produce amorphous material. In this thesis, lactose has been successfully rendered amorphous by freeze-drying. NMR, polarimetry and thermal methods were able to fully characterise the material produced. It has been shown within this thesis that the β/α anomeric content of a dried amorphous lactose sample depends on the standing time of the feed solution. Therefore, a recommendation from the work reported here to produce a consistent anomer composition within spray- and freeze-dried amorphous lactose is that the standing time for the feed solution should be greater than 4

h. Thus, the feed solution is well-removed from the most dynamic region of the epimerisation profile and so an equilibrium content of 63% β and 37% α is achieved.

Lactose is a ubiquitous ingredient for a whole host of pharmaceutical and confectionary formulations (McSweeney *et al.*, 2009; Franke *et al.*, 2008; Pifferi *et al.*, 1999; Fox *et al.*, 1998; Timsina *et al.*, 1999; Khankari *et al.*, 1995; Wade *et al.*, 1994). The work reported in this thesis will aid workers in the field allowing them to understand not only the impact of time but also the influence of temperature on the epimerisation kinetics rate of lactose. This thesis has reported that the rate of epimerisation of α-lactose to β-lactose dramatically increases with temperature. For example, the overall rate constant at 25 °C has increased from $k = 4.08 * 10^{-4}$ sec^{-1} to $k= 35.01* 10^{-4}$ sec^{-1} at 60 °C. This information is important in the context of spray-drying process as it involves exposure of the sample to high temperatures (~ 90 °C and above). The results described in chapters 2 & 3 indicate that the actual spray-drying process is quicker than the epimerisation kinetics since the standing time still influences the β/α ratio within the spray-dried products. The generated results have shown that the preparation of the lactose solutions at 4 °C minimises epimerisation. The calculations reported in chapter 3 show that at 4 °C, the half-life ($t_{1/2}$) of an aqueous solution of α-lactose reaching anomeric equilibrium is 128.4 min. This gives an indication of the working window of preparation of aqueous lactose solutions at 4 °C especially that it was also shown that it takes 3 min before a significant 1% degradation takes place at 4 °C. In the polarimetric work reported here, solutions were prepared at 4°C and analysed with 3 minutes within the polarimeter. Once in the polarimeter, the temperature equilibrated very rapidly to its set value, within a minute, because the path length of the sample cell was 2 mm, and the temperature was controlled by a peltier device. When working at high temperatures e.g. 60 °C, the half-life of lactose solutions is 3.3 min which indicates that the analytical measurement must commence very quickly after the preparation of the lactose solution.

Such information is considered highly beneficial to both industrial applications and academic research scientists as it suggests a handling procedure of lactose solutions especially that in most of the literature published concerning lactose, no information or details about the solution standing time have been reported. This gives a lack of confidence of the generated data as the properties of the starting feed material, even when working at different temperatures, were not well known (Listiohadi *et al.*, 2009; Haque *et al.*, 2005; Ramos *et al.*, 2005; Buckton *et al.*, 2002; Chidavaenzi *et al.*, 1997; Roetman *et al.*, 1975). Furthermore, this work proposes a methodology which other workers in the field can apply on other sugars to investigate their corresponding epimerisation rate constants.

Sucrose can undergo inversion to produce its invert sugars glucose and fructose (Mathlouthi *et al.*, 1995). Amorphous sucrose was successfully produced by freeze-drying. Thermal analysis proved to be effective in characterising the material produced and polarimetry had shown that no inversion occurred during the freeze-drying cycle.

The work reported here shows that sucrose is stable in aqueous solutions. Such stability makes sucrose ideal for calibrating polarimeters. Moreover, the methods developed for the characterisation of sucrose can be transferred to other sugars e.g. dextrose, fructose and trehalose which are commonly found in confectionary products and in freeze-dried pharmaceuticals. It is worth mentioning here that sucrose samples purchased from different suppliers possessed different physical properties, i.e a different melting profile. Therefore when considering characterising and formulating sucrose, the origin of the sucrose should be always clearly stated.

During the chocolate crumb manufacturing process, the sugars are exposed to water, heat, mixed vigorously and then a vacuum applied (Harris, 2012, personal communication). For example, when the ingredients of crumb are mixed, sucrose and lactose are present in a system which contains approximately 25% water. The mixture is then exposed to high temperature and vacuum. During this stage, as the mixture reaches supersaturation, crystallisation commences

producing a suddenly stiff mixture that causes a high increase in the power needed to operate the reactor. This power draw is significantly increased when minerals are also present. This observation was the driver for studying in more depth the interactions between the individual sugars on each other's behaviour and the interaction with the minerals that might be present (Harris, 2012, personal communication).

As the crumb system is complex (containing sugars, minerals, whey proteins, milk, cocoa butter), a model system was designed to minimise any complexity that might occur during data analysis. The model crumb contained sucrose and lactose as major ingredients and minerals and water vapour as minor components. Thereafter, a design of experiment (DoE), described in chapter 5, was applied to the model system.

The obtained results showed that NaCl was able to trap more water within the freeze-dried sucrose cake, this result agrees with what is reported in literature by Santagapita *et al.*, 2008 and Chen *et al.*, 2005. Moreover, NaCl was able to reduce the re-crystallisation enthalpy of amorphous sucrose, which also agrees with literature reports (Santagapita *et al.*, 2008; Telang *et al.*, 2003; Hartel, 1991; Shalaev *et al.*, 1996). This is due to the tendency of NaCl to keep the water in hydration shells surrounding its mineral ions, thus making it unavailable for aiding in the re-crystallisation process. This happens due to the charge density and balance forces e.g. electrostatic and hydrogen bonds around the minerals (Hribar *et al.*, 2002; Her *et al.*, 1995). The high content of residual water led to crystallisation occurring at a lower temperature. This can be attributed to water increasing molecular mobility which is required for molecular reorganisation and thus successful crystallisation (Labuza, 2004; Makower *et al.*, 1956). It was also shown that lactose interacts with sucrose, the assumption is that this interaction takes place via hydrogen bonds and this interaction inhibits crystallisation. This has been observed in the model system where at even medium lactose concentrations, crystallisation is completely inhibited. These results agree with what is reported in literature about the effect of lactose on the crystallisation of sucrose (Anzai *et al.*, 2011; Labuza *et al.*, 2006; Arvanito *et al.*, 2004; Kedward *et al.*, 1998).

Although many workers have spent a long time characterising T_g in order to characterise the materials physical stability, one of the significant observations reported in the DoE work is that small changes in composition and sample presentation did not affect the T_g. However, these parameters had a dramatic impact on crystallisation.

An important experiment to consider is that the NMR β/α anomeric ratio, during DoE, was determined for lactose (in presence of sucrose). It was found that the anomeric ratio didn't change from what was previously obtained for pure lactose, (chapter 2). This means that the presence of sucrose did not affect the final anomeric equilibrium ratio of lactose and consequently this led to the elimination of one extra variable during the DoE process. Because if the β/α anomeric ratio of lactose in the model system was different from that obtained for lactose alone, then the reduction or even inhibition of re-cystallisation might have been attributed to the difference in the β/α anomeric ratio.

The DoE is a useful tool when studying mixed systems and can have various applications in the pharmaceutical and confectionery fields. For example, the granulation process uses small amounts of water where issues similar to the crumb making process may arise, production of an amorphous phase and subsequent re-crystallisation may occur. Thus, the DoE approach described here may be translated to granulation process. Furthermore, DoE is useful for scientists involved in the development of freeze-dried dosage forms as this gives them a better understanding of the interactions taking place between the different components of the feed solution which consequently have an impact on the final formulated product (Her *et al.*, 1995). Moreover, DoE can help to avoid issues arising from unanticipated crystallisation from mix systems during the shelf-life of the product (Saleki-Gerhardt *et al.*, 1994).

6.2 Conclusion

The research aims of the project have been fulfilled. The impact of standing time on β/α was established. Sucrose in solution and in its amorphous freeze-dried form was shown to be

relatively chemically stable, when compared to lactose. However, there are significant differences in the physical properties in the crystal forms of sucrose from different suppliers. The importance of T_g appears to be lowered when investigating re-crystallisation in mixed sugar containing matrices. The work presented here has been able to provide an explanation for the thermally induced changes observed in crumb reactor, i.e. the presence of water or minerals lowers the temperature of crystallisation by reduces the amount of crystallisation observed.

6.3 Future work

Future work should focus on developing model systems which are closer to real chocolate. This will determine whether the analytical techniques developed within this thesis are applicable to complex systems like chocolate and will determine the feasibility of translating the findings of chapters to real chocolate samples.

Further investigations of the attained findings about the epimerisation kinetics of lactose at different temperature; for example, expanding the temperature range of the Chirascan experiments, will be considered. This will involve conducting Chirascan experiments at temperatures lower than 25 °C and higher than 60 °C. Moreover, there is a a growing interest in developing a method for calculating the final β/α anomeric equilibrium at 45 °C and 60 °C. This might be done by running the Chirascan experiments in DMSO and titrating with different amounts of water e.g. 1 %, 2 %, 3 %. Furthermore, investigating the epimerisation kinetics of other sugars can also be considered.

The future work that would compliment the data reported with respect to the impact of different parameters on the crystallisation of sucrose will involve the control of ratios of amorphous to crystalline forms of sugars during both crumb chocolate processing. This will also reflect on the powder recipes, roles of amorphous sugars on structure and impact on processbility (including that of milk powders and derivatives), chocolate intermediates and impact on mouth feel of the chocolate.

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Appendix I: Certificate of analysis of lactose

SIGMA-ALDRICH®

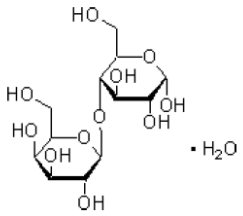
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Product Specification

Product Name:
α-Lactose monohydrate - ≥99% total lactose basis (GC)

Product Number:	**L3625**
CAS Number:	5989-81-1
MDL:	MFCD00150747
Formula:	C12H22O11 · H2O
Formula Weight:	360.31 g/mol



TEST	Specification
Appearance (Color)	White to Off-White
Appearance (Form)	Powder
Solubility (Color)	Colorless
Solubility (Turbidity) 100 mg/ml, H2O	Clear
Impurity (by Enzymatic) Glucose	≤ 0.05 %
Purity (GC) Total-Lactose	≥ 99 %
Impurity Beta-Lactose	≤ 4 %
Recommended Retest Period 4 years	----------

Specification: PRD.1.ZQ5.10000007338

1 of 1

Appendix II: Certificate of analysis of sucrose

Fisher Chemical

Specification Sheet

Product: Sucrose | **Code: S/8600/60** | **Grade: AR, ACS**

Application: For analysis | **Expiry Phrase: Use within 5 yrs of opening**

Test	Guaranteed	Units
Acidity/alkalinity (meq/g)	≤ 0.0008	meq/g
Calcium (Ca)	≤ 10	ppm
Copper (Cu)	≤ 1	ppm
Insoluble matter (%)	≤ 0.005	%
Invert sugar	≤ 0.05	%
Iron (Fe)	≤ 1	ppm
Lead (Pb)	≤ 1	ppm
Loss on drying	≤ 0.03	%
Magnesium (Mg)	≤ 5	ppm
Potassium (K)	≤ 20	ppm
Reducing sugars (%)	≤ 0.005	%
Residue after ignition	≤ 0.01	%
Sodium (Na)	≤ 50	ppm
Specific rotation	≥ 66.4 and ≤ 66.6	Degrees
Total chloride (Cl)	≤ 0.001	%
Total nitrogen (N)	≤ 20	ppm
Total phosphorus (P)	≤ 20	ppm
Total silicon (Si)	≤ 5	ppm
Total sulfur (S)	≤ 20	ppm
Zinc (Zn)	≤ 10	ppm

Additional Information: This product conforms to BP, ACS and EP specifications

Mrs K S Cluskey BSc
Quality & Compliance Manager.

Sunday 22nd of April 2012 03:42:34 PM
Any queries please phone: +44(0)1509 555158

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